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January—March, 1961

Number 1

# INDIAN JOURNAL OF MICROBIOLOGY

*Edited for the Association of Microbiologists of India*

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in association with

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# INDIAN JOURNAL OF MICROBIOLOGY

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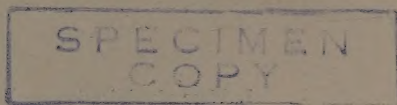
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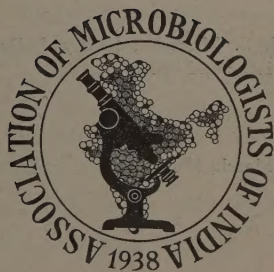
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## FOREWORD

The underlying unity of concept and purpose in all aspects of the study of micro-organisms is now universally recognised. Nevertheless, the diversity of fundamental and practical knowledge which microbiology comprehends has always been an impediment to its development as a unified scientific discipline. This may be seen from the constant alternation of the processes of accumulation of new knowledge and its synthesis into fruitful and unifying concepts which mark its history. In recent years biochemistry, the scientific foundations of which derive so largely from studies of fermentation processes, has in turn furnished impressive demonstration of the essential unity of microbiology. While micro-organisms still continue to be among the most rewarding experimental material for biochemical investigations, advances in biochemical knowledge are being continuously applied to furthering the understanding and resolving the complexity of microbial processes and phenomena.

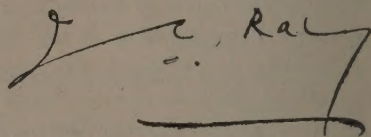
The rise of biophysics and intensification of research at the molecular biological level gives promise of further and accelerated increase of both factual and conceptual knowledge. We may look forward in the coming years to rapid growth of knowledge in several aspects of microbial study, such as the molecular basis of genetic phenomena and reproduction of cellular constituents, modification of genetically determined metabolic processes, and fine structure in relation to replication, growth and nutrition. This will profoundly influence techniques and ideas in the different branches of applied microbiological research like medicine, agriculture, animal husbandry, fermentation technology and antibiotics.

There is consequently continuing need for keeping in focus an integrated view of microbiology as a scientific discipline and presenting in their proper perspective original contributions to microbiological knowledge over the wide front it covers to-day. It is primarily to serve this end that the Association of Microbiologists of India have instituted publication of the *Indian Journal of Microbiology*. The journal aims to bring together within its covers the results of microbiological research in India in all the divergent and specialised directions it is taking. If thereby it succeeds in enlivening the fruitful interaction of thought and ideas in the different specialities of microbiological study, it will indeed have served its purpose well.

It has been the long-cherished desire of the Association of Microbiologists of India to establish a journal of their own. We have now taken the first step, but there is a long period

of striving ahead to achieve the highest standards of scientific journalism we have set as our goal. Despite our best efforts, there are bound to be shortcomings and deficiencies in the earlier issues in the scientific quality and presentation of the papers, as well as in format, typography and lay-out. However, with sustained and earnest effort, it should be possible to remedy these gradually and justify our experiment in scientific journalism by its success. For the present, I would request the scientific world to judge this journal by its efforts to improve rather than by its actual level of achievement.

In the past, when science received but meagre support in the country, and a tradition of scientific research, particularly in biology, was still in the making, India had workers whose names have gone down in the history of microbiology. Their example and inspiration should fortify us in our effort to establish the *Indian Journal of Microbiology* as a significant organ of microbiological thought and knowledge.

A handwritten signature in black ink, appearing to read "J. C. Ray". The signature is stylized, with a long horizontal line extending from the left and a large, sweeping "Z" shape on the right.

# INDIAN JOURNAL OF MICROBIOLOGY

Vol. I, No. 1, 1961

## THE ENZYMATIC HYDROLYSIS OF AMIDES BY *SALMONELLA TYPHOSA*

S. GHATAK AND V. K. MOHAN RAO

From the Central Drug Research Institute, Lucknow

(Received for publication, November 1959)

In course of our investigations on the enzyme make-up of *Salmonella typhosa* we showed that this micro-organism was capable of deaminating the common amino acids, specially aspartic and glutamic acids (Ghatak *et al.*, 1958). Since asparaginase and glutaminase play an important role in biological systems in the conservation of ammonia, it was considered worthwhile to investigate and characterise the amidases present in this organism. These two enzyme systems have not been studied extensively in bacteria, even though it has been shown that asparaginase is present in *S. enteritidis* (Saito, 1941), autolysates of certain strains of bacteria (Uttjino and Imaizumi, 1938; Busch, 1948) and glutaminase in *Proteus morganii* (McIlwain, 1948), *Clostridium welchii* (Krebs, 1948 a,b); Hughes and Williamson, 1952) and *Escherichia coli* (Hughes, 1949). However no literature is available in this regard with reference to *S. typhosa*.

In view of this, preliminary studies have been made on the enzymatic hydrolysis of asparagine and glutamine by resting cells as well as the cell-free extracts of the virulent strain (Ty2) of *S. typhosa* and the results are communicated in this paper.

### MATERIALS AND METHODS

#### *Enzyme Preparation*

Cell suspension of the Ty2 strain of *S. typhosa* was prepared by the method described earlier (Ghatak, 1960). Cell-free extract was prepared either by grinding the frozen cells with abrasives and extracting with normal potassium chloride (KCl) solution or by subjecting the KCl suspension of the cells to ultrasonic treatment for 20 min. in a Mullard Sonic Oscillator using 25 Kc/S frequency and 2.5 ampere output current, followed by high speed centrifugation (10,000 r.p.m.). The extract thus obtained can be stored at  $-20^{\circ}\text{C}$  for a long time without much loss of activity.

#### *Assay of the Enzyme Activity*

The procedure for determining the asparaginase and glutaminase activities involved the estimation of the corresponding free amino acid moieties, liberated after the hydrolysis, employing descending paper partition chromatography. For this purpose Whatman No. 1 filter paper sheets (18"  $\times$  22") were run at room temperature (25-30°C) in a 'Chromatocab' using phenol saturated with water as the mobile phase. The ninhydrin stained amino acid spots were estimated, according to the method of Giri *et al.* (1953) using Klett Summerson photoelectric colorimeter at 540 m  $\mu$ .

Control was simultaneously run with each experiment and due allowance was made while presenting the results.

## RESULTS

With a view to screening the presence of the deamidase activities in *S. typhosa*, aliquots of enzyme preparation (cell suspension or cell-free extract) were incubated at 38°C with M/15 phosphate buffer (pH 7.0) and freshly prepared asparagine (M/50) or glutamine (M/20) solution. Experiments were also run using aspartic and glutamic acids as substrates to ascertain the presence of the deaminase activities in these enzyme preparations. The enzyme activity was stopped by boiling the reaction mixture after 2 hr. on a water bath for 5 min. 0.02 ml. of the same were spotted on the filter paper and the chromatogram was run in the usual way.

Results of these experiments indicated that the resting cells of *S. typhosa* could readily deamidate asparagine and glutamine and deaminate the corresponding amino-acids. However, the cell extract could liberate the amino-acid moieties from both the amides, while the corresponding amino-acids were not significantly deaminated. From this it would appear that cell extract contains only the amidase activity. It was also observed that the amidase activity of the cell-extract was nearly double that of the intact organism.

The optimal conditions for the deamidation of L-asparagine and glutamine by the sonicates of 20 hr. old growth of *S. typhosa* were studied.

### *Effect of pH*

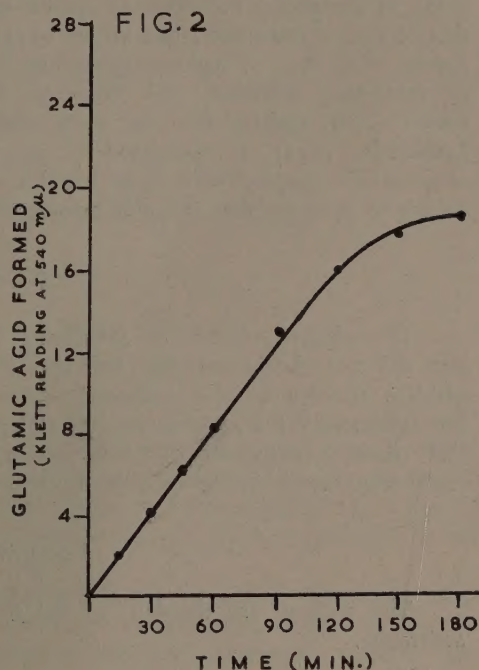
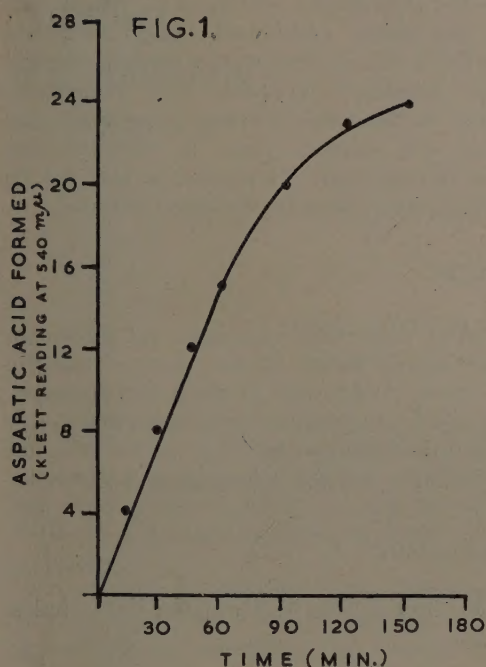
The asparaginase and glutaminase activities of Ty2 strain were determined at various pH levels using M/5 phosphate buffer of 5.5–8.5 pH range. The results would indicate that these enzymes possess sharp peaks at pH 6.5 and 7.0 respectively.

### *Time activity relationship*

The time activity curves showed that direct proportionality existed between the length of incubation and the amino-acid moiety liberated by enzyme action. The state of saturation was reached within 2 hr. with both the amides (Figs. 1 and 2.)

### *The effect of activators and inhibitors*

The action of a number of metal ions, metal binding agents and certain organic and inorganic reagents on the hydrolysis of asparagine and glutamine by *S. typhosa* was investigated in order to distinguish these systems from the amidases of other sources. The incubation mixture consisted of 0.5 ml. sonicate of Ty2 strain, 1.0 ml. phosphate buffer pH 7.0 and 2 micromoles of the activator or inhibitor. The incubation was done for 30 min. at 38°C and then the reaction was run as usual for 2 hr. with the two substrates. The final concentration of these compounds in the reaction mixture was 0.66 micromole per ml. The results would show that Cu<sup>++</sup>, Zn<sup>++</sup>, Co<sup>++</sup>, fluoride and cyanide inhibited both asparaginase and glutaminase activities, while iodoacetate and the metal binders were not significantly active. Hydrolysis of glutamine was activated by sulphate and arsenate, whereas arsenate alone stimulated the asparaginase system.



Time activity curves showing the amino-acid moiety liberated by enzyme action.

#### DISCUSSION

The present study indicates that the intact cells of *S. typhosa* deamidate asparagine and glutamine as well as deaminate the corresponding amino acids; while the cell extract possesses only deamidase activity. The high amidase activity in cell extract when compared to that of the intact cells may probably be explained on the basis of the permeability factor of the cell membrane. It is also noticed that longer spinning at higher speeds (20,000-25,000 r.p.m.) did not significantly alter the enzyme activity. Hence it is possible that the enzyme systems are not associated with the granular fractions of the bacterial cells.

In the present study the organism showed maximum asparaginase and glutaminase activities at pH 6.5 and 7.0 respectively. It may be mentioned here that the optimum pH of *S. typhosa* asparaginase coincides with that of *S. enteritidis* (Saito, 1941) and is some what less than the yeast (Grassmann, and Mayr, 1933) and other microbial asparaginases (Utgino and Imaizumi, 1938); whereas the optimum pH of glutaminase of *S. typhosa* differs widely from those of *P. morganii* (McIlwain, 1948), *C. welchii* (Hughes and Williamson, 1952) and *E. coli* (Hughes, 1949) which are active between pH 4.0-5.0. Kidney glutaminase is, however, active at pH 7.2-7.5 (Archibald, 1944a).

The characterisation of the two enzyme systems is attempted by studying the effect of metal ions, metal binders and certain enzyme inhibitors and activators. As a

result of comparing the effect of cyanide on the asparaginase activity of *S. typhosa* with that of yeast (Grassmann, and Mayr, 1933) and kidney (Archibald, 1944b), it would appear that the *S. typhosa* asparaginase is more akin to that of the kidney, wherein an inhibitory influence was observed by Archibald (1944a). The glutaminase activity of *S. typhosa*, like the same enzyme in the tissue extracts (Greenstein and Leuthardt, 1948), is stimulated by arsenate and sulphate ions. In view of these observations coupled with those based upon the optimum pH studies, it may not be wrong to presume that the glutaminase of *S. typhosa* resembles the tissue enzyme.

#### SUMMARY

The cell-free extract of *Salmonella typhosa* deamidated asparagine and glutamine, but did not deaminate the corresponding amino acids. It was observed that the amidase activity of the cell-extract was nearly double that of the intact organism. The optimum pH for the hydrolysis of asparagine and glutamine was between 6.5-7.0. The characterisation of the two enzymes was attempted by studying the effect of metal ions, metal binding agents and certain common enzyme inhibitors and activators.

#### ACKNOWLEDGMENT

The authors are thankful to Sri S. K. Bose and Sri V. Varma for the technical assistance.

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# INDIAN JOURNAL OF MICROBIOLOGY

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## NUCLEOLYTIC ENZYMES OF *SALMONELLA TYPHOSA*

S. GHATAK

*From the Central Drug Research Institute, Lucknow*

(Received for publication, November 1959)

*Salmonella typhosa* is an ideal choice for studies on enzymic reactions, since it is one of the few pathogens possessing well-defined and stable antigenic characteristics. Strains H-901, O-901 and Vi1 of *S. typhosa* contain predominantly pure H, O and Vi antigen respectively while Ty2 and Watson's V contain a combination of H, O and Vi antigens. O antigen is held responsible for toxicity and the Vi for virulence of the organism.

A systematic investigation has been undertaken in our laboratory to study qualitative and quantitative differences of enzyme activities in strains of *S. typhosa* in order to throw light on the relationship between antigenic structure and enzyme content. The presence of deaminases (Ghatak *et al.*, 1958), dehydrogenases (Ghatak, 1960), glycerophosphatase (Shrivastava *et al.*, 1954), oxidases (Shrivastava *et al.*, 1953, 1954), aldolase (Ghatak and Shrivastava, 1958), catalase (Ghatak *et al.*, 1958), and organic nitro-reductase (Ghatak and Shrivastava, 1959) in this organism has already been shown. In the present paper nucleolytic enzymes, hydrolysing nucleic acid and its derivatives, are reported.

### MATERIALS AND METHODS

Twenty hr. growth of *S. typhosa* at 37°C on beef heart infusion agar, pH 7.4, was harvested with normal KCl solution and the turbidity of the suspension adjusted to 20% in Lumetron photo-electric colorimeter (650 mμ).

The cell-free extract was prepared by grinding the frozen cells with abrasives and extracting with cold 0.9% KCl followed by spinning at 46,900 × g for 20 min. The straw coloured transparent supernatant was stored at -20°C. It could depolymerise ribonucleic acid (RNA) and readily attack the phosphoric acid ester linkage of purine and pyrimidine nucleotides but did not possess nuclein deaminase activity.

### RESULTS

#### *Ribonuclease*

Assay by Mac Donald's (1955) method showed significant amounts of ribonuclease (RNase) in the cell-free extracts of *S. typhosa* strains, in the following order: Ty2, Vi1, H-901, Watson's V and O-901. RNase activity could not be detected in fresh cell

suspensions. The enzyme activity of the extract did not change appreciably on storing under frozen condition for seven days. All these strains showed optimal activity at pH 7.0 and a substrate concentration equivalent of 0.75 mg/0.5 ml.

Amongst the metal ions tried only  $\text{Cu}^{++}$  and  $\text{Co}^{++}$  strongly inhibited cell-free RNase activity of Ty2 strain. The inhibitory action of  $\text{Cu}^{++}$  and  $\text{Co}^{++}$  on RNase from other sources has been reported by Zittle (1946) and Miura and Nakamura (1951).  $\text{Ca}^{++}$  and  $\text{Mn}^{++}$ , which are reported to be inhibitory for pancreatic RNase in 0.5  $\mu$  mole concentration (Mac Donald, 1955), stimulated the same enzyme of *S. typhosa*.

Semicarbazide, hydrazine sulphate and iodoacetic acid displayed no significant action on *S. typhosa* RNase while 8-hydroxyquinoline,  $\alpha$ - $\alpha'$  dipyridyl, *o*-phenanthroline and ethylene diamine tetra acetic acid (EDTA) strongly activated the enzyme activity.

### Nucleotidase

The dephosphorylation of nucleotides was assayed by incubating resting cells or cell-free extract with veronal buffer and specified nucleotides (1  $\mu$  mole) at 37°C and estimating the inorganic phosphate released in 2 hr. by Fiske and Subbarow's (1925) method. The cell-free extract was slightly less active than the intact cells and partially lost its enzyme activity within 72-96 hr. when stored at -20°C. The activity was demonstrable in all the strains, Ty2 possessing the maximum and Watson's V relatively the least dephosphorylating activity. The enzyme system was not specific, as it could dephosphorylate a number of nucleotides. Of the six nucleotides tested adenosine triphosphate and muscle adenylic acid showed minimum cleavage while uridylic, cytidylic and yeast adenylic acids were metabolized at a faster rate.

The enzymes have a sharp optimum pH at 7.0 and the dephosphorylation of the substrates was a linear function of time upto a period of 1 hr. The rise in activity was proportional upto 0.5  $\mu$  mole substrate concentration.

$\text{Zn}^{++}$  and  $\text{Cu}^{++}$  completely inhibited the nucleotidase activity while  $\text{Co}^{++}$ ,  $\text{Mg}^{++}$  and cyanide had stimulating effect. Sodium fluoride, semicarbazide and iodoacetate did not have significant action on this enzyme system. Of the metal binding agents only EDTA was strongly inhibitory while  $\alpha$ - $\alpha'$  dipyridyl and *o*-phenanthroline activated *S. typhosa* nucleotidase.

### Nuclein deaminase

The deamination of several derivatives of nucleic acid was assayed by a method described earlier (Ghatak *et al.*, 1958). Resting cells of all the strains of *S. typhosa* could deaminate a number of purine and pyrimidine compounds, Vi1 showing the maximum activity closely followed by Ty2, H-901 and O-901 strains while Watson's V was least active. The cell-free extracts, in general, showed very little enzyme activity. The rate of deamination was seen to vary from substrate to substrate. Adenosine, cytidine and cytidylic acid were well deaminated while adenylic acid, adenine, guanine and guanosine were comparatively less active. Similar pattern of results has been obtained by Agarwala *et al.* (1954) in *Vibrio cholerae*.

The optimum pH for the nuclein deaminase in case of all the substrates was found to be around 7.0. There was a linear relationship between activity and the quantity of the enzyme used in the reaction mixture as well as between the extent of substrate

decomposed with the length of incubation. Ca, Ni and Zn chlorides and sodium fluoride had no significant action while  $\text{Co}^{++}$  and iodoacetate strongly activated the enzymes in minute concentrations. Iodoacetate, however, was reported to inhibit the adenosine deaminase activity of the vibrios (Agarwala *et al.*, 1954). All the metal binding agents, glutathione, arsenite and cyanide inhibited the deamination of adenosine and cytidylic acid in the order mentioned.

### DISCUSSION

*S. typhosa* has been shown to be equipped with enzymes capable of depolymerising ribonucleic acid, dephosphorylating uridylic, cytidylic and adenylic acids and deaminating a number of nucleotides, nucleosides and purine bases. Ribonuclease is present only in the cell-free extracts, nuclein deaminases are mainly present in the intact cells while nucleotidases are present in both. The lack of ribonuclease activity in the cells is probably due to the impermeability of ribonucleic acid across the cell membrane. Similar lack of action of bacterial cells on yeast ribonucleic acid has been noticed in *Escherichia coli* (Mason, 1953) and *V. cholerae* (Sagar *et al.*, 1958). The slight deamination of the purine and pyrimidine compounds by the extracts of *S. typhosa* would suggest that the nucleo-deaminases are probably associated with the particulate fractions of the bacterial cells.

In all the enzyme systems studied, only quantitative differences have been observed amongst the five standard strains of *S. typhosa*, Ty2, the most virulent strain containing all the three antigens, always exhibited the highest activity. However, no significant relationship existed between antigenic structure and enzyme content. The optimum pH for all the enzyme systems was around the neutral pH which is the best pH for the growth and proliferation of *S. typhosa*.

Like other ribonucleases, the enzyme from *S. typhosa* is stable to heat and storage but the nucleotidase activity of the extract is partially lost when stored for three to four days even under frozen conditions.

From the results presented it is clear that the three enzyme systems reacted differently to the action of metal ions, metal binding agents and a few recognized enzyme inhibitors and activators on the individual enzymes of *S. typhosa*. Among the metallic ions copper strongly inhibited all the three enzyme systems while cobalt inhibited only RNase activity of *S. typhosa*. The strong activation of RNase by the metal binders probably suggests that these reagents remove certain inactivating cations from the site of action by chelation. The lack of action of iodoacetate on the RNase and nucleotidases of the organism would indicate that active—SH groups may not be necessary for the normal functioning of these enzymes.

### SUMMARY

The standard antigenic strains of *Salmonella typhosa*, H-901, O-901, Vi1, Ty2 and Watson's V, depolymerise ribonucleic acid, dephosphorylate uridylic, cytidylic and adenylic acids, and deaminate purine and pyrimidine compounds in the following order: adenosine, cytidine, cytidylic, adenylic acids, adenine, guanine and guanosine. Ribonuclease is present only in the cell-free extract while nucleodeaminases are mainly

associated with the intact cell. Nucleotidases are present in both. In all the enzyme systems studied, no correlation could be obtained between antigenicity and enzyme content. The optimum pH for all these enzyme systems has been found to be around 7.0. Attempts have been made to characterise the individual enzymes by studying the effect of a few cations, metal binders and recognised inhibitors and activators on the cell-free extract of *S. typhosa*.

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## RHIZOSPHERE BACTERIAL FLORA OF WHEAT AND BERSEEM

W. V. B. SUNDARA RAO AND M. V. CHAYANULU

*From the Indian Agricultural Research Institute, New Delhi*

(Received for publication, November 1959)

Lochhead and his colleagues have made important contributions on the bacterial flora of the rhizosphere by publishing, during the years 1938 to 1957, fifteen papers under the general title of "Qualitative Studies of Soil Micro-organisms" (Canada Department of Agriculture, Ottawa Publication, 1959). The subject of soil bacteria and growth-promoting substances has been reviewed recently by Lochhead (1958). The present study deals with the bacterial population of the rhizosphere of wheat (*Triticum vulgare*) and berseem (*Trifolium alexandrinum*).

### MATERIALS AND METHODS

#### *Pot-culture experiment (Rabi, 1957-58)*

Thirty lb. of Delhi soil were put in each glazed pot (9" diameter and 12" height) and the experiments were set up by the method of Desai and Sundara Rao (1948). The analysis of the soil showed 0.045% N, 0.00063% available  $P_2O_5$  (Olsen *et al.*, 1954) and 0.35% organic carbon. The soils in the pots were treated as in Table I. Twelve healthy berseem and five wheat (N.P. 797) seedlings were kept in each pot and the moisture content of the soils was maintained at about 12%.

TABLE I.  
*Manurial treatment of the pot-culture soil*

Treatment	Symbol	Berseem			Wheat		
		N	$P_2O_5$ lb./acre	$K_2O$	N	$P_2O_5$ lb./acre	$K_2O$
1. Unmanured	O	—	—	—	—	—	—
2. Ammonium sulphate + triple superphosphate + muriate of potash	NPK	40	100	30	60	60	20
3. Farmyard manure (applied 20 days before sowing)	FYM	100	70	8	100	70	8
4. Triple superphosphate + muriate of potash	PK	—	100	30	—	—	—

### *Field experiment*

In the experiments where the residual effects of fertilizers on berseem were studied, the treatments were: (1) no manure and (2) sulphate of ammonia 100 lb., superphosphate 120 lb and sulphate of potash 120 lb./acre. In the direct effect of fertilizer studies the treatments were: (1) 20 lb. N as sulphate of ammonia and (2) 20 lb. N as sulphate of ammonia and 60 lb.  $P_2O_5$  as superphosphate/acre. The wheat plots selected were those receiving no manure, NPK and farmyard manure.

### *Collection of soil sample*

Plants selected at random were carefully removed with roots intact. The soil clumps were broken to remove the superfluous soil. The soil adhering to the roots was collected by shaking the roots. The control soil was collected by the method of Katznelson and Chase (1944). The soil samples were air-dried, passed through a 2.5 mm. sieve and bottled.

In the case of pot-culture experiments with berseem, two sets of soil samples were collected, one two months after sowing and the other at the time of last cutting. One set of soil samples was collected two months after sowing in the case of wheat.

In field experiment, where the direct effect of fertilizer was studied on berseem and wheat crops, one sample was collected from each field. One set of soil samples was collected 150 days after sowing of berseem, and another at the time of final cutting from the field where the residual effect of fertilizer was studied.

### *Bacterial count*

Thornton's (1922) agar medium was used for plate counts. Soil extract agar, as used by Lochhead and Chase (1943), resulted in the development of too many spreaders which interfered with the counting and also suppressed the development of other colonies. The counts were taken on the tenth day after incubation at 32°C because the maximum number of colonies developed between the 7th and the 10th day.

The amino-acid requiring organisms were counted by plating on Thornton's agar to which twenty-three amino acids, as suggested by Wallace and Lochhead (1950), were added.

In the count of micro-organisms from a soil sample, mean of the count of four plates was taken.

## RESULTS

Judging from the nitrogen, organic carbon and the available  $P_2O_5$  contents of the soil used in pot-culture experiments, the soil seems to be deficient in available  $P_2O_5$ . The carbon-nitrogen ratio was 8:1. Considering the responses in crop yields to the manurial treatments, both nitrogen and phosphorus were deficient in the soil (Fig. 1). The overall mean counts of bacteria in the case of both wheat and berseem were significantly higher in farmyard manure treatment over those in no-manure treatment. The treatment with NPK increased the bacterial count in the case of berseem only (Fig. 1).

The rhizospheres of berseem and wheat supported a larger bacterial population than the control soil (Fig. 1).

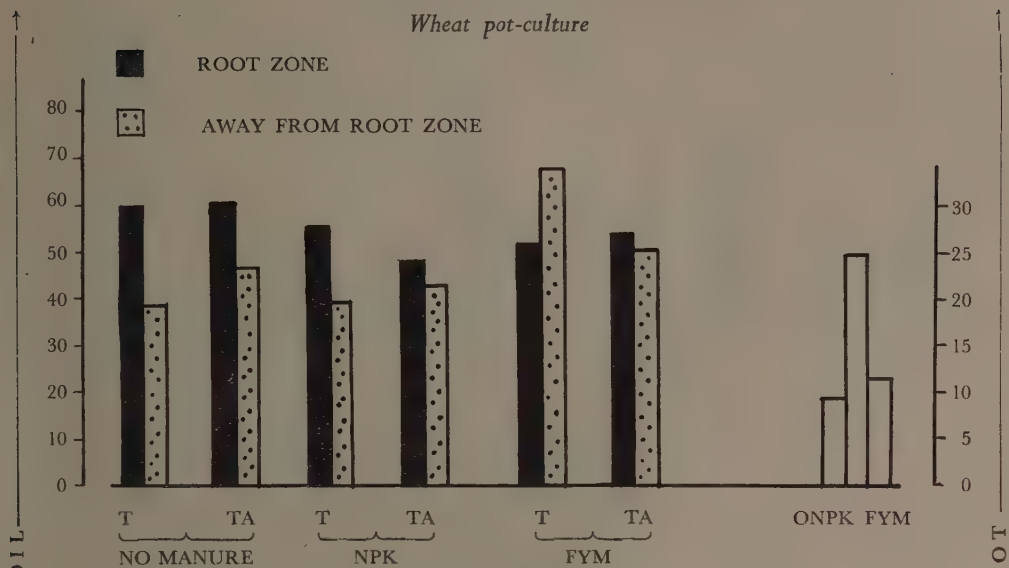
*Wheat pot-culture*

Fig. 1(a)

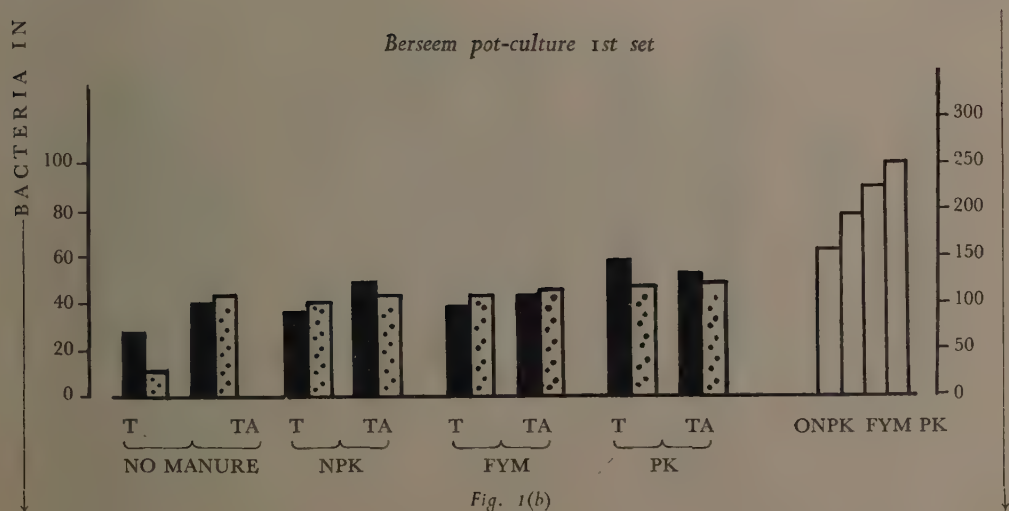
*Berseem pot-culture 1st set*

Fig. 1(b)

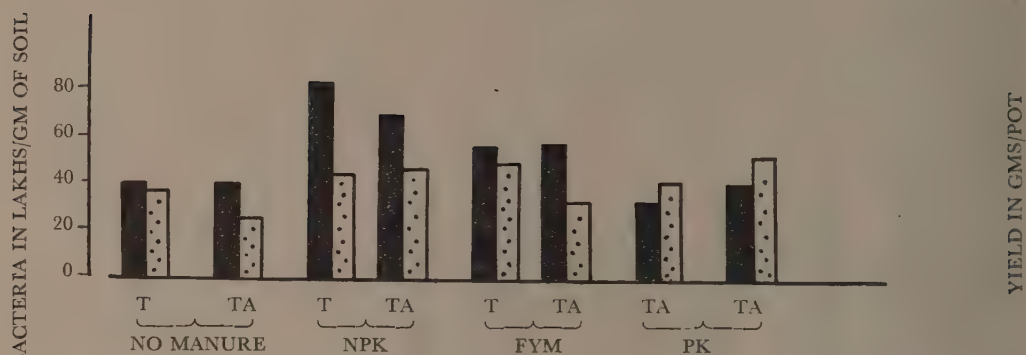
*Berseem pot-culture 2nd set*

Fig. 1(c). Count of bacteria from soil. T=Thornton's agar; TA=Thornton's agar + amino-acids.

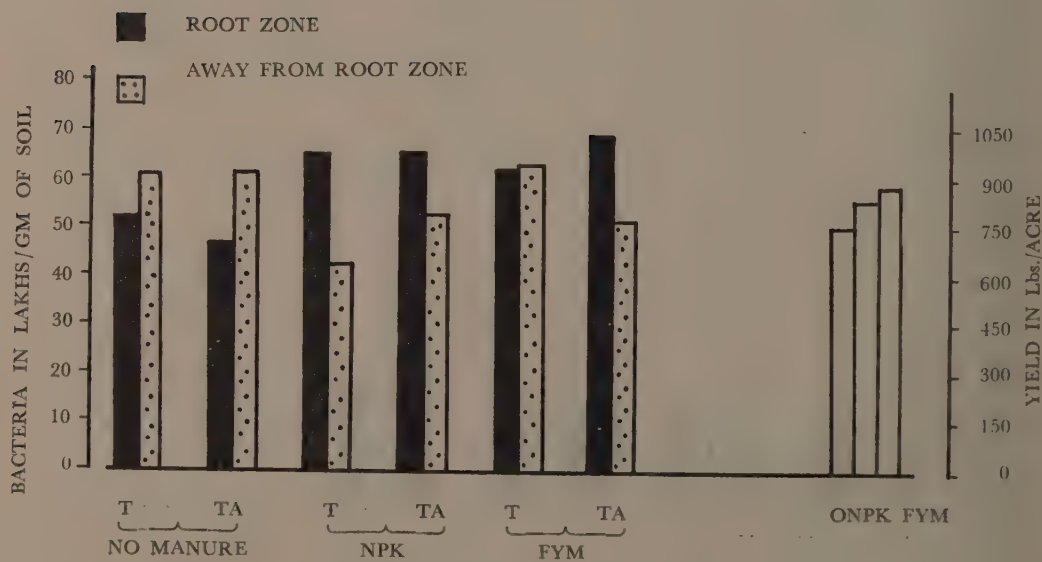
*Wheat-field crops*

Fig. 2(a)

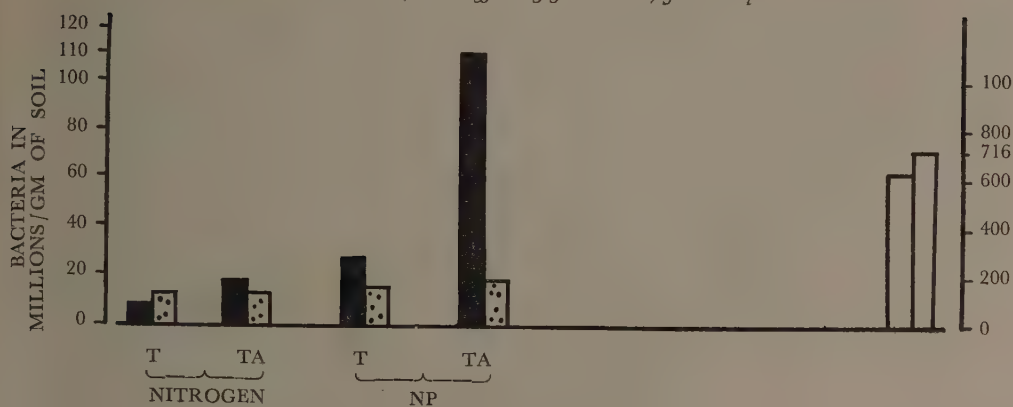
*Berseem (direct effect of fertilizers)-field crop*

Fig. 2(b)

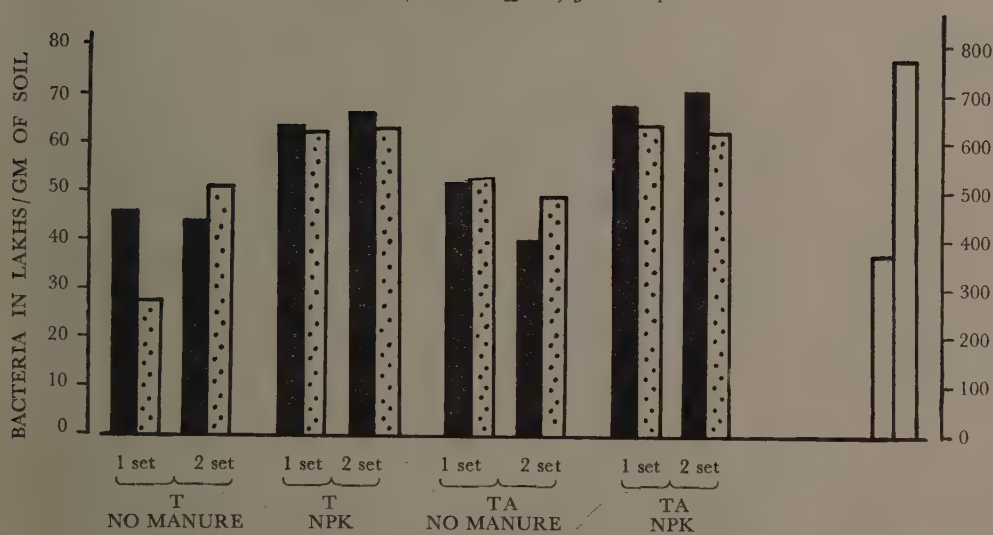
*Berseem (residual effects)-field crops*

Fig. 2(c). Count of bacteria from soil, T=Thornton's agar ; TA=Thorntons agar + amino-acids,

In the berseem field experiment, under direct effect of fertilizers, there was a significant increase in the counts of bacteria in the rhizosphere (Fig. 2). The overall mean bacterial count were  $41.5 \times 10^6$  for rhizosphere and  $16.62 \times 10^6$  for the control, C.D. @5% level being 12.31. This is in accord with the observations of Waksman and Starkey (1931), Thom and Humfeld (1935) and Waksman (1952) that the roots of higher plants exert beneficial effects on the micro-organisms in the soil adhering to the roots, resulting in an increase in their population. The bacterial population was higher in NP treatment than that in N treatment only. The difference in the population in the rhizosphere and control soil was more marked in NP treatment than in N treatment. Further, a higher bacterial count was obtained in Thornton's agar + amino-acids than in Thornton's agar alone. In the rhizosphere significantly higher count was obtained in Thornton's agar + amino-acid medium than in Thornton's medium alone, while the corresponding values in the control did not differ significantly (Fig. 2). From this it would appear that the rhizosphere of berseem supported some organisms having special requirements of amino acids and as such could not grow in Thornton's agar medium alone. This suggests that the berseem roots excreted amino-acids which supported this type of micro-organisms. Similar results were obtained by West and Lochhead (1940). Biswas and Das (1957) observed a predominance of aspartic acid in the soil where berseem was grown. It is probable that the beneficial effects of berseem in building up soil fertility, as judged by the yields of succeeding crops (Parr and Bose, 1944; Desai, Sundara Rao and Tejwani, 1953) might be partly due to this phenomenon. In the experiment with berseem crop, where the residual effects of fertilizers were studied, a higher bacterial count as well as higher yield were obtained in NPK over those in no-manure treatment in both the sets of soil samples (Fig. 2). In this experiment the bacterial counts in the rhizosphere were not significantly higher than those in the control.

Bacterial counts in soil samples from wheat field experiments showed in general no significant difference between the treatments or between the two media or in the overall effects of rhizosphere (Fig. 2). Only in the NPK treatment, the rhizosphere supported a larger bacterial population. The yield data of wheat crop were not found to be significantly different among the treatments.

In Table II is given the counts of actinomyces and organisms forming colonies with clear zones suggesting the production of antibiotics. The actinomyces count was highest in soil samples taken from berseem plots under direct effects of fertilizers. In pot and field experiments, berseem crop supported a higher population of organisms with clear zones in the rhizosphere than wheat crop in the no-manure and NPK treatments. However, in pot-culture experiments, the difference in counts was not significant between FYM-treated soils where wheat and berseem were grown. In the case of wheat crop organic manuring increased bacterial population as compared to no-manure and NPK treatments.

#### SUMMARY

In pot-culture experiments there was a higher bacterial population in the rhizosphere than in the control soil, supporting berseem and wheat crops. In field experiments a similar result was obtained in the case of berseem crop. With wheat crop, it was only in the NPK treatment that the bacterial count was higher in the rhizosphere. Where

TABLE II  
*Counts of actinomycetes and organisms forming colonies with clear zones (soil samples collected in April, 1958)*

Pot-culture experiment				Field experiment			
Berseem		Wheat		Berseem (direct fertilizer effect)		Wheat	
Actino- myces	Count 100,000/g. soil	Count 100,000/g. soil	Count 100,000/g. soil	Count millions/g. soil	Count 100,000/g. soil	Count 100,000/g. soil	Count 100,000/g. soil
Treatment	Root zone	Control	Treatment	Root zone	Control	Treatment	Root zone
No manure	4.5	5.3	No manure	—	3.7	No manure	2.7
NPK	2.3	4.5	NPK	—	5.5	NPK	2.7
FYM	7.7	6.9	FYM	—	9.7	FYM	2.0
Colonies with clear zones							
No manure	3.5	4.5	No manure	—	1.0	No manure	—
NPK	2.0	5.0	NPK	3.0	2.0	NPK	—
FYM	4.2	5.7	FYM	—	4.0	FYM	2.7
PK	2.7	3.0					4.5

the effect of direct application of fertilizers was studied in field with berseem, a higher count of amino-acid requiring organisms was obtained in the rhizosphere. Berseem soil fertilized by N or NP in the field supported a higher actinomyces population than wheat. The former crop under residual effect of fertilizers, supported a higher population of antibiotic-synthesising organisms than wheat crop in no-manure and NPK treatments. Organic manuring increased their counts in soil.

#### ACKNOWLEDGMENT

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## LABORATORY STUDIES ON ENTEROPATHOGENIC *ESCHERICHIA COLI* SEROTYPES\*

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Diarrhoea in infants and children is an important problem in India because of the high rates of morbidity and mortality. In the Christian Medical College Hospital, Vellore, relatively few specific diagnoses with respect to *Shigella* and *Salmonella* species were being made and it was decided to investigate the problem of etiology of the diarrhoeas.

After considering the evidence which had accumulated in other parts of the world implicating certain serotypes of *Escherichia coli*, it seemed likely that these organisms might be the etiological agents of diarrhoea in our patients also, particularly in the neonatal population from whom we had repeatedly failed to isolate *Shigella* or *Salmonella* species. Therefore a study was planned for the purpose of detecting so called enteropathogenic *E. coli* in our patients.

As far back as 1927, Adam as cited by Neter (1951) suggested on the basis of absence of recognised pathogens and presence of certain fermentative types of *E. coli* that these might have etiological significance, but the development of serological procedures marked the advance necessary for the definite establishment of this. Work such as that of Taylor and others (1949, 1952, 1957, 1958, 1959) in Great Britain, Kauffmann (1954), Ørskov (1951, 1954, 1955, 1959) and others as cited by Kauffmann (1954) in the Scandinavian countries, and Edwards and Ewing (1955) and others (1951, 1952, 1953, 1954, 1955) in the U.S.A. accomplished this and to-day every one is convinced of the etiological role of *E. coli* in diarrhoea.

Though many different serotypes of *E. coli* have been incriminated as the causative agents in infantile and neonatal diarrhoea attention was focussed in our study on only five, namely 0111:B4, 055:B5, 026:B6, 086:B7 and 0127:B8. Of these the first three, as

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\* The studies reported here formed a part of the work done by Dr. Bhat in the Department of Microbiology, Christian Medical College and Hospital, Vellore, S. India, in partial fulfilment of the requirements of the University of Madras for the M.Sc. Degree and were included in the dissertation presented in August, 1959, under the title, "Studies on the Etiology of Diarrhoea in Children with particular reference to the pathogenic serotypes of *Escherichia Coli*".

stated by Taylor (1958), are found to be pathogenic all over the world, whereas certain other strains, including the last two are only intermittently found associated with disease.

#### MATERIAL AND METHOD

The study to be reported in this paper was carried out on strains of *E. coli* isolated during an eleven and half-month period which began in December 1957. Stool specimens, were received as rectal swabs from the neonatal babies in the maternity wards and also older infants admitted into the children's ward or attending outpatient clinics. Most specimens came from infants with symptoms of diarrhoea. However, as the presence of enteropathogenic *E. coli* serotypes in healthy contacts who remain asymptomatic is a well recognised fact, as many as possible of the newborns free from symptoms of diarrhoea but who had been exposed to infection were also checked and included as "Controls" in the study.

Specimens from a total of 105 cases of diarrhoea among neonatal babies and 399 among older infants and children were examined for the detection of pathogenic *E. coli* serotypes and also *Shigella* and *Salmonella* species. In addition, 289 new born babies were examined as "Controls" as well as 25 children of an older age group.

For collection of the specimens special emphasis was laid on the following points: (1) material for culture was to be collected before any antibiotic therapy was begun; (2) specimens collected for study were to be very fresh when sent to the laboratory.

The media used primarily were MacConkey Agar (MA) and Sheep Blood Agar (BA) both prepared in our own laboratory. The reason for using a partially selective medium like MA was that the growth of *E. coli* serotypes is generally poor on the more highly selective media which are usually employed for the isolation of *Salmonella* and *Shigella* species. BA was used as it gave an indication of the kinds of other organisms present, e.g. *proteus*, *staphylococci*, *streptococci*, etc. Besides this, in some instances *E. coli* serotypes associated with infantile diarrhoea may appear on BA and not grow on MA, though in this study they grew on both media equally well. In addition, the haemolytic activity exhibited by some strains of *E. coli* could be noted.

All plates were inoculated in such a manner as to get isolated colonies and then incubated at 37°C for 18-20 hr. after which they were examined for colonies of *coli*-form bacteria. No specific characteristics aid the selection of colonies likely to be those of *E. coli* serotypes found associated with infantile diarrhoea, for they appear quite similar to those of non-pathogenic strains; but generally colonies which are large, mucoid and raised like those of *Klebsiella*-*Aerobacter* can be excluded from consideration. Colonies, which were opaque and smooth, with regular entire edge, were then picked up for immediate serologic typing by slide agglutination and for subsequent biochemical and further serologic studies. Usually about 8-10 discrete colonies and the sweep from the confluent growth were investigated before reporting the specimens as negative for pathogenic *E. coli*.

Before going into the procedure for serologic typing it may be worthwhile to include a few remarks on the B antigens generally found in the enteropathogenic *E. coli* serotypes. B antigens belong to the group of K antigens and occur as envelopes which may mask the O antigen. They are heat-labile and hence the agglutinability of B antigen in B

antiserum can be destroyed by heating at 100°C for  $\frac{1}{2}$  to 1 hr. which unmasks the O antigen and renders the coli suspension agglutinable in O antiserum. Antigenicity is inactivated by such treatment but antibody binding power is not. The main test for the presence of a B antigen is inagglutinability of the living bacteria (in smooth form) in O antiserum and their agglutinability with OB serum.

For purpose of serologic typing a polyvalent antiserum containing the O and B agglutinins for *E. coli* serotypes 0111:B4, 056:B5, 026:B6, 086:B7 and 0127:B8 was used and also the respective specific OB and O serums of all these five serotypes. In the earlier part of the study, OB serums given to us by Kauffmann and Ørskov were used; but in the latter part serums prepared in our own laboratory were used. The Kauffmann strains obtained through the kindness of Dr. Kauffmann were used for immunization of rabbits and titres obtained in our animals were comparable to those of the State Serum Institute (Denmark) serums. The O serum used was purchased from the Difco Laboratories in U.S.A.

The following technique for slide agglutination was used. A portion of an individual colony of *E. coli* suggestive of the pathogenic types was emulsified in a drop of sterile normal saline. This suspension was considered satisfactory for typing if there was no autoagglutination. A drop of *E. coli* polyvalent OB antiserum properly diluted was then added to this suspension by means of a wire loop measuring 3 mm. in diameter and the mixture was stirred vigorously by the same wire loop.

The almost instantaneous appearance of a coarsely granular macroscopic agglutination was taken to indicate a positive reaction and to provide presumptive evidence that one of the five serotypes was present in the specimen.

Following this the particular serotype was determined as follows. The remaining portion of the colony tested, a little of the agglutinated bacteria from the slide, and 3-4 similar looking colonies were subcultured on BA slopes and also on MA plates. Following incubation, the growth was re-tested with all the individual OB antisera by the slide agglutination method.

When living suspensions of freshly isolated strains of the pathogenic *E. coli*, for example 0111:B4 or 055:B5, were tested in their homologous OB antisera, agglutination occurred rapidly and usually was complete. This was due to the inter-action of B antigen and B antibody. This was, in effect a rapid screening test and gave an idea of the B antigen of the particular organism under test, but O antigens were also determined. For this the growth on the blood agar slopes was washed off with a little normal saline, and the suspension heated at 100° C for  $\frac{1}{2}$  hr. to destroy the B antigen. After cooling, it was re-tested in the same antiserum by the slide method. When the tube method of agglutination was to be used, suspensions were heated for 1 hr. as prescribed by Edwards and Ewing (1955).

Results obtained by slide agglutination were confirmed by carrying out a test tube agglutination using a formalinised suspension and a heated suspension for B and O antigen respectively, and the specific OB serum. Usually a titre of 1-500 for O antigen and 1-160 to 1-320 for B antigen was taken as confirmatory. So also agglutination of the unheated suspension and heated suspension was tested for in the respective O serum by slide and tube method. With the unheated suspension, no agglutination was noticed whereas with the heated there was agglutination. The inhibiting action of the surface (B) antigen over the somatic (O) antigen in the unheated suspension which resulted in

inagglutinability of O antigen in its specific O serum was destroyed; and the unmasking resulted in agglutination.

Following serologic typing a study of the biochemical reactions and antibiotic sensitivity patterns was carried out.

Indol, Methyl Red and Voges-Proskauer reactions were determined in the classical manner, with use of peptone, high in tryptophane content, and glucose phosphate broth. Simmon's Citrate Agar was used for the test of ability to utilize inorganic carbon. These reactions are generally described as the *IMViC* formula. The  $H_2S$  production was determined in Triple Sugar Iron Agar in which glucose, sucrose, lactose and ferrous sulphate are incorporated. Urease production was determined by using urea broth.

The carbohydrate broths included were glucose, lactose, sucrose, maltose, mannite and sorbitol. Inoculated carbohydrates were generally held for 15 days unless fermentation occurred in a shorter time.

Motility was determined by growth in semisolid agar deeps prepared according to a formula devised in our own laboratory.

The test for susceptibility to different antibiotics was carried out by the paper disc method using penicillin, ilotycin, aureomycin, achromycin, chloramphenicol, terramycin, streptomycin and neomycin, according to Myers and Achaya (1956), though neomycin was not included in their study.

## RESULTS

In most cases of acute diarrhoea it was found that the particular *E. coli* serotype which was responsible for the disease grew virtually in pure culture. Such colonies were few in cultures from the healthy carriers. This might have at times resulted in failure to detect carriers in spite of careful search and examination. Tables I and II summarise the findings among neonates.

TABLE I  
*Incidence of enteropathogenic E. coli among neonates*

Clinical status	Neonates		
	Number Studied	<i>E. coli</i> positive	
		Number	%
Diarrhoea	105	42	40.0
No diarrhoea	289	29	10.0
Total	394	71	18.0

Enteropathogenic *E. coli* was isolated from 71, or about 18 percent, of 394 newborn babies. Of these 105 were diarrhoeic cases and 289 were "Controls". Forty-two of the 105, or 40 percent of the diarrhoeic cases, were positive for pathogenic *E. coli*, and 29 of the 289, or 10 percent of the "Controls", were positive for pathogenic *E. coli*.

Strains of 055:B5, the most frequently isolated serotype, comprised 87.3 per cent of the total strains isolated. Of the 71 positive neonatal babies, 62 were found to be

TABLE II

*Incidence of serotypes among 71 enteropathogenic E. coli strains isolated from neonates*

Serotype	Diarrhoea	No diarrhoea
055:B5	40	22
086:B7	2	6
0127:B8	0	1
026:B6	0	0
0111:B4	0	0
Total	42	29

excreting 055:B5. Forty of these had frank diarrhoea and 22 were healthy contacts. Among the total 105 diarrhoea cases approximately 40 percent were associated with this type.

The second predominant type was 086:B7. Eight of the 71 positive neonatal babies were found to be excreting 086:B7, among whom 6 were healthy carriers and 2 were diarrhoeic cases, Serotype 0127:B8 was isolated only once and then from a non-diarrhoeic baby. The other two serotypes 0111:B4 and 026:B6 were not isolated from any of the 394 neonatal babies.

Tables III and IV summarise the findings among infants of 1 month and above.

TABLE III

*Incidence of enteropathogenic E. coli among infants of 1 month and above*

Clinical status	Infants of 1 month and above		
	Number studied	<i>E. coli</i> positive	
		Number	%
Diarrhoea	399	22	5.5
No diarrhoea	25	0	0
Total	424	22	5.2

Enteropathogenic *E. coli* was isolated from 22 cases in the group of 399 diarrhoeic children one month in age and above, an incidence of 5.5 percent. Here also 055:B5 was the predominant type, comprising 14, or 63.6 percent of the strains isolated. The second most common type was 026:B6, with 4. Types 086:B7 and 0127:B8 were isolated twice and 0111:B4 was isolated only once. Though the incidence for the entire group was only 5.5 percent, it was of interest that within the group, the incidence was greatest among infants below 12 months of age and least among children above 3 years. Considering along with this observation the 40 percent positive incidence among neonates, it seems that there is a direct correlation between age and susceptibility to infection: the younger, the more susceptible. Examinations on 25 healthy infants and children of older age groups did not show any positive carriers.

TABLE IV

*Incidence of serotypes among the 22 positive older infants and children*

Serotype	Infants 1 month in age and above	
	Diarrhoea	No diarrhoea
055: B <sub>5</sub>	14	0
086: B <sub>7</sub>	2	0
0127: B <sub>8</sub>	2*	0
026: B <sub>6</sub>	4	0
0111: B <sub>4</sub>	1	0
Total	23	0

\* One associated with 055: B<sub>5</sub> also. Hence 23 isolations from 22 cases of diarrhoea.

A study of certain biochemical reactions and antibiotic sensitivity patterns of all the strains isolated revealed some important points of epidemiological significance. The sugars included were lactose, sucrose, glucose, maltose, mannite and sorbitol, as stated previously. Though these were not such an extensive series of carbohydrates as recommended by the Enterobacteriaceae Subcommittee (30), for the establishment of fermentation types, it was found that all of the 72 strains of 055: B<sub>5</sub> available for study could be broadly divided into two different types with reference to maltose fermentation. (Type 055: B<sub>5</sub> was isolated 76 times but we were able to study only 72 isolates as 4 strains were lost.) Table V shows these reactions.

Some 055: B<sub>5</sub> strains fermented maltose within 24 hr. with acid and gas and others gave a negative reaction or a marked delayed fermentation. There were 36 strains which gave negative or delayed fermentation, and 36 strains fermented this sugar rapidly. These were designated as Fermentation Type I and Type II respectively. Lactose, glucose and mannite were fermented by all the 72 strains within 24 hr. with acid and gas. Sucrose also was fermented within 24 hr. with acid and gas except by 17 strains which, though they fermented sucrose with acid within 24 hr., produced gas only within 48-72 hr. Sorbitol fermentation was usually a delayed reaction, but 4 strains did not ferment sorbitol at all.

The IMViC reaction was that of typical *faecal coli*, “+ + — —”, i.e.: Indol positive, Methyl Red positive, Voges-Proskauer negative, no growth in Citrate. All strains were urease-negative and H<sub>2</sub>S-negative. No other biochemical tests were done.

All the 72 strains of 055: B<sub>5</sub> were moderately sensitive to neomycin and resistant to penicillin and ilotycin. With regard to the remaining antibiotics these strains could be broadly divided into (1) those which were resistant to all and (2) those which showed varying combinations of sensitivity and resistance. These findings are also given in Table V.

In comparing antibiotic sensitivity patterns and fermentation activities it was observed that the strains which were moderately sensitive to neomycin and resistant to other antibiotics were irregular fermenters of maltose, i.e. they were Fermentation Type I strains. Strains which were moderately sensitive to neomycin and moderately or slightly

TABLE V

Biochemical reactions and antibiotic sensitivities of 72 strains of *E. coli* serotype 055:B5

	Fermentation Types	
	I	II
Glucose, Lactose, Mannite	++	++
Sucrose	++	++*
Maltose	x	++
Sorbitol	+L†	+L†
Indol	+	+
M — R	+	+
V — P	—	—
Simmon's citrate	—	—
H <sub>2</sub> S	—	—
Urease	—	—
R to all antibiotics except neomycin to which MS	+	—
MS to neomycin, R to penicillin and ilotycin and MS or SS to others	—	+
No. of Strains	36	36

\* 17 strains fermented sucrose with acid within 24 hr.  
and gas only within 48-72 hr.

† Few strains were sorbitol negative.

++ Acid and gas within 24 hr.

x Irregular, late or no fermentation.

L Delayed fermentation.

+ Positive.

— Negative.

MS Moderately sensitive.

R Resistant.

SS Slightly sensitive.

sensitive to other antibiotics fermented maltose within 24 hr. *i.e.* they were Fermentation Type II strains. Thus there seemed to be a correlation between antibiotic activity and fermentation activity.

Further, it was observed that the various strains of *E. coli* serotype 055:B5 isolated in the nurseries up to the month of July belonged to Fermentation Type I and strains isolated from August to the end of November belonged to fermentation type II indicating that two different strains of *E. coli* serotypes 055:B5 were responsible for the outbreaks of diarrhoea in the nurseries during these different periods. An agglutination absorption test to further confirm this observation was not attempted.

Among the older children, 13 strains of 055:B5 isolated at varying times throughout the entire study came under Fermentation type II. The fourteenth strain which was Fermentation Type I was isolated during the first part of the study, but from a 10-month old baby who had not been born in our hospital.

The possibility of using antibiotic sensitivity test patterns to detect differences in strains isolated so as to trace epidemiological relationships has been discussed by Ørskov

(1951). Lie Kian Joe *et al.* (1958) in Indonesia observed that though strains of a particular *E. coli* serotype showed the same biochemical reactions, this did not necessarily mean that only one strain was implicated and the antibiotic sensitivity patterns showed differences of epidemiological significance.

All the ten strains of 086:B7 isolated during the study fermented glucose, lactose, mannite, sucrose and maltose within 24 hr. with acid and gas, and 5 fermented sorbitol with acid and gas within 24 hr.; the other five were late fermenters of sorbitol. The IMViC formula was “++--” and urease and H<sub>2</sub>S production were negative. No correlation between fermentation types and antibiotic sensitivity patterns such as observed with 055:B5 was found, but this series was comparatively a much smaller one.

Serotype 0127:B8 strains were isolated 3 times, twice from cases of diarrhoea and once from a healthy contact. It was found that the 2 strains from diarrhoeic cases were more like the strains described by Ewing *et al.* (1955) in that they were late sucrose and sorbitol fermenters and that indol production was delayed and weakly positive. These two strains were moderately sensitive to all the antibiotics except penicillin and ilotycin to which they were resistant. One strain of 0127:B8 was found associated with 055:B5.

There were only four strains of type 026:B6 isolated, all from diarrhoeic cases, one associated with *S. flexneri*. Of these, 3 showed definite haemolysis on blood agar (sheep and human). Strain F 41, the test strain of 026:B6 supplied by Kauffmann, which was reported to be non-haemolytic when grown by Ørskov and Taylor (1959) on horse blood, showed definite haemolysis when grown in our laboratory on sheep blood and human blood agar. Reexamination of this strain and also of some of the C.M.C. Hospital isolates sent to Denmark has been carried out by Dr. Ørskov who in a personal communication reported confirmation of our findings. One of our strains failed to ferment lactose, mannite and maltose and would have been missed but for the haemolysis on BA. Among the other 3 strains 1 fermented all the sugars within 24 hr., 1 fermented all except sucrose; and the remaining one fermented all within 24 hr. excepting sucrose which was fermented late. The IMViC formula for all strains was “++--”. All were urease- and H<sub>2</sub>S-negative. The antibiotic sensitivity pattern was different for each of them.

The only strain of 0111:B4 isolated fermented all the sugars within 24 hr. except sucrose which was fermented within 2 days. This strain was very sensitive to aureomycin and terramycin, moderately sensitive to achromycin, streptomycin and neomycin and slightly sensitive to chloramphenicol and resistant to penicillin and ilotycin.

Most of the serotypes on primary isolation were non-motile, though a few strains were sluggishly motile. No attempt was made to study the H antigens.

#### SUMMARY

1. The occurrence of enteropathogenic *E. coli* among neonates and children aged one month and above, with particular reference to serotypes 0111:B4, 055:B5, 026:B6, 086:B7 and 0127:B8, was investigated during the period December 1957—November 1958.

2. The laboratory procedures used, with emphasis on serologic typing, have been presented. Stool specimens were received as rectal swabs, with multiple specimens

when possible and were inoculated on MacConkey Agar and Blood Agar plates. For purposes of serologic identification both slide agglutination and test tube agglutination were carried out using procedures to detect both O and B antigens.

3. One hundred and five cases of diarrhoea among neonates and 399 cases of diarrhoea among infants 1 month of age and above were studied with the following results: a) Serotype 055:B5 was the most commonly isolated strain in both the groups: 38 percent among neonates and 3.5 percent among older infants. (b) The other serotypes were isolated less frequently: 086:B7 2 times from neonates and 2 times from older infants; 026:B6 4 times from older infants; 0127:B8 twice, and 0111:B4 once, from older infants.

4. The existence of asymptomatic healthy carriers among the neonatal population was demonstrated by the examination of 289 healthy contacts, in 7.9 percent of whom 055:B5 was found; in 2.1 percent, 086:B7, and in 0.35 percent, 0127:B8.

5. Specimens from 25 older children were examined. None was found to be positive for enteropathogenic *E. coli*.

6. From specimens of diarrhoeic cases the serotype responsible grew virtually as a pure culture, whereas colonies in culture from carriers were few in number. For this reason, though a careful search was made, it may be that a few carriers were not detected.

7. Some strains of 026:B6 isolated in this study showed definite haemolysis, a finding not previously reported.

8. The biochemical reactions, though limited, and the antibiotic sensitivities of the strains isolated from our patients showed that, based on maltose fermentation, the 72 strains of 055:B5 studied could be divided into two fermentation types which correlated with two antibiotic sensitivity patterns. Epidemiologic significance was indicated in that during two distinct outbreaks first one of these types was isolated from the patients concerned and then the other.

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# INDIAN JOURNAL OF MICROBIOLOGY

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## INFLUENCE OF SOIL SOLUTION ON NITROGEN FIXATION BY *AZOTOBACTER* *sp.*

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(Received for publication, November 1959)

Inorganic salts have been known to have remarkable influence on nitrogen fixation by *Azotobacter* *sp.* (Erdman, 1923; Albrecht, 1930; Burk and Lineweaver, 1931, 1940; Webb, 1949; Greaves and Anderson 1936; Jensen, 1954) As different soils contain different amounts of soluble salts, it was considered of interest to study the effect of water extracts of soils on nitrogen fixation by *Azotobacter* *sp.*

### MATERIALS AND METHODS

Surface (0.9") soils from Shahjahanpur, Travancore, Coimbatore, Banaras, Rohtak, Nagpur, Kanpur, Ferozepur, Poona, Pusa and Delhi were air dried and passed through a 2 mm. sieve before the extracts were made. In the cold extraction, 1 kg. of soil was shaken with a litre of CO<sub>2</sub>-free distilled water in an end to end shaker for half an hr. and then the solution was filtered. In the hot extraction, 1 kg. of soil was treated with a litre of CO<sub>2</sub>-free distilled water and heated in an autoclave at 15 lb. pressure for half an hr. cooled and filtered. The composition of the soil extracts are given in Tables I and II Phosphorus, iron and manganese contents were determined by colorimetric

TABLE I.  
*Composition of the soil extracts by hot extraction*

Soil locality	Mineral matter*	Organic carbon*	Loss on ignition of the residue*
Sahajahanpur	0.080	0.0018	0.036
Travancore	0.732	0.0360	0.312
Coimbatore	0.080	0.0018	0.052
Banaras	0.100	0.0060	0.020
Rohtak	0.080	—	0.020
Nagpur	0.080	—	0.020
Kanpur	0.092	0.0168	0.020
Ferozepur	0.120	0.0024	0.040
Poona	0.060	0.0030	0.008
Pusa	0.180	0.0102	0.080
Delhi	0.140	0.0072	0.060

\* Expressed in g./100 ml. of the extract

TABLE II.

*Iron, phosphorus and manganese content of soil extracts by cold and hot extractions*

Soil locality	Iron*		Phosphorus*		Manganese*	
	Hot	Cold	Hot	Cold	Hot	Cold
Sahajahanpur	0.08	0.08	4.24	Nil	Tr.	Tr.
Travancore	Nil	Nil	13.50	7.25	0.40	0.30
Coimbatore	0.02	Nil	25.50	Nil	1.05	0.30
Banaras	0.14	0.14	9.20	Nil	0.35	0.60
Rohtak	0.10	0.14	17.50	Nil	0.30	0.40
Nagpur	0.04	0.04	11.75	Nil	0.55	0.60
Kanpur	0.06	0.06	10.25	Nil	1.00	Tr.
Ferozepur	Nil	Nil	14.25	Nil	Tr.	Tr.
Poona	Nil	Nil	5.25	Nil	Tr.	Tr.
Pusa	Nil	Nil	19.25	Nil	0.90	0.75
Delhi	Nil	Nil	4.75	Nil	Tr.	Tr.

\* Expressed as parts per million; Tr. = Trace

method of Piper (1950) and organic carbon by chromic acid oxidation method of Walkley and Black (1934).

A strain of *Azotobacter* sp., isolated from a Delhi soil, was selected because of its wide range of pH tolerance. The characteristics of the organism appear elsewhere (Sundara Rao and Iswaran, 1959). Fred and Waksman's (1928) minerals medium No. 77 was used in the experiments. The nitrogen fixing capacity of *Azotobacter* sp. was 5.83 with a range of 5.5-6.2 mg./g. of mannite in the original medium. Mo in Jensen's (1951) medium enhanced nitrogen fixation sufficiently as to mask the effect of small quantities of salts in the soil extract. The inoculated media were incubated at 32°C for three weeks after which the increase in nitrogen content was determined. The nitrogen fixation determinations were done in three replications.

## RESULTS

Nitrogen fixed by *Azotobacter* sp. in water extracts of soils obtained by hot and cold extractions is given in Table III.

Both hot and cold extracts of soil stimulated nitrogen fixation by *Azotobacter*. Extracts obtained by hot extraction gave higher fixation than those from cold extraction. Presence of phosphorus in hot extraction (Table II) may be one of the causes of higher fixation, except in the case of Travancore soil.

Statistical analysis of the data in Tables I and II, with the exception of peaty and abnormally acid soil of Travancore, shows a highly significant negative correlation with the mineral matter content and a highly significant positive correlation with organic carbon content of the soil extracts on nitrogen fixation. The negative correlation confirms the earlier observations of Iswaran and Sen (1958) that salinity depressed nitrogen fixation by *Azotobacter* sp.

The relationship between nitrogen fixed and the mineral matter content of the soil extract could be expressed as  $y = 10.18 - 1.38 x$ , where  $y$  is the nitrogen fixed in mg./g. of mannite and  $x$ , the amount of mineral matter in g./100 ml. of the extract (Fig. 1). Similarly, the relationship between the amount of nitrogen fixed and the organic carbon contents of the soil extract could be expressed as  $y = 10.60 + 130.80 x$ , where  $y$  is the amount of nitrogen fixed and  $x$ , the organic carbon content of the soil extract in g./100 ml. (Fig. 2).

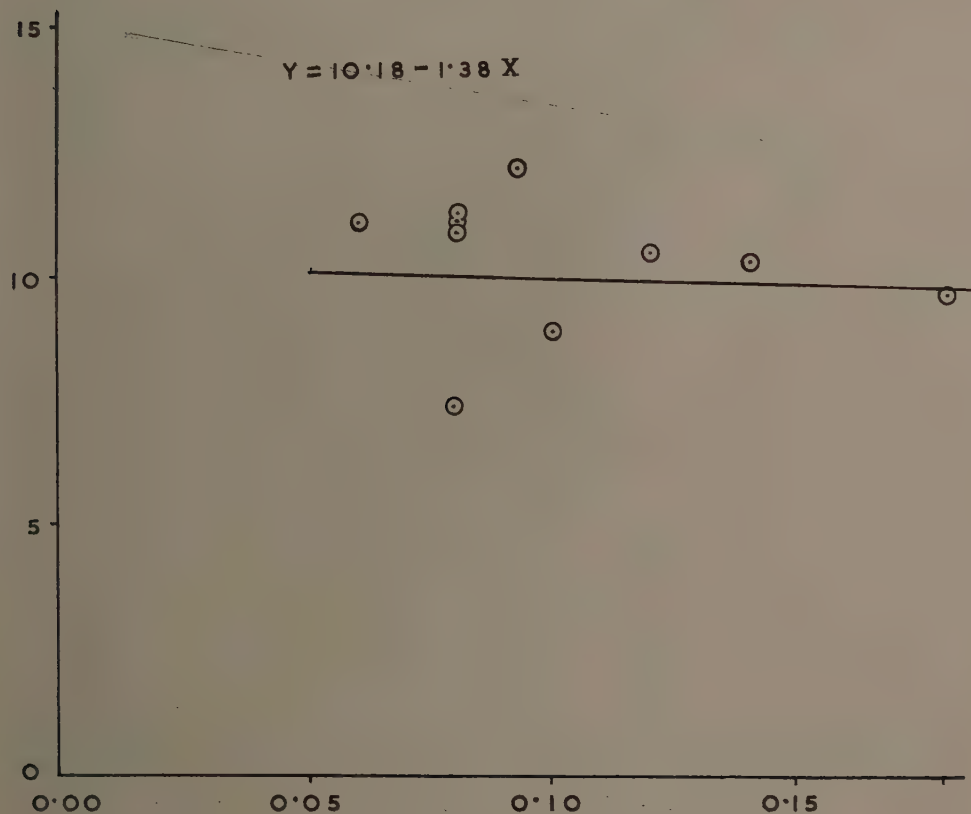


Fig. 1. Relationship between nitrogen fixed and mineral matter content of the soil extract.

These observations are supported by an experiment carried out with a single soil extract obtained by the hot extraction from a Delhi soil. A portion of the soil extract was boiled with  $H_2O_2$  to destroy organic matter and evaporated to dryness. The residue was treated with water and the original volume of the extract was made up with additions of distilled water. Different volumes of the two soil extracts, one unaltered and the

other free of organic matter were added to the minerals and energy material of the medium. Nitrogen-fixation in these media is given in Table IV. The increase in the concentration of soil extract in the medium resulted in increased nitrogen-fixation.

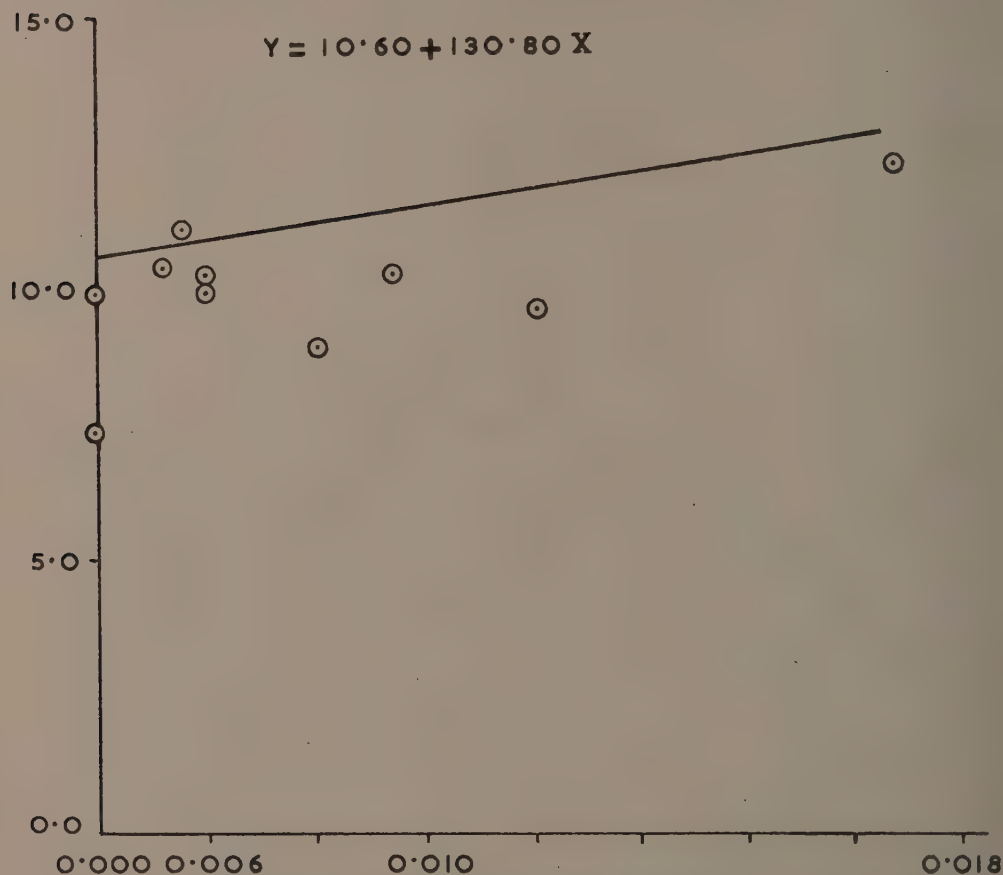


Fig. 2: Relationship between nitrogen fixed and carbon content of the soil extract.

From Figs. 1 and 2, it could be seen that organic matter in solution had a stronger stimulating effect than the depressing effect of inorganic salts. This was observed also in the nitrogen-fixation in the organic matter free soil extract, where not much change in nitrogen fixation was found with increasing quantities of organic matter free soil extract.

TABLE III.

*Effect of soil extract on nitrogen fixation*

Soil locality	Nitrogen fixed in extract* (hot extraction) mg./g. of mannite		Nitrogen fixed in extract* (cold extraction) mg./g. of mannite	
	Average	Range	Average	Range
Sahajahanpur	10.10	9.9—10.3	8.53	8.5—8.6
Travancore	3.80	3.7—4.0	—	—
Coimbatore	10.20	9.9—10.4	8.43	8.4—8.5
Banaras	9.03	9.0—9.1	8.63	8.6—8.7
Rohtak	9.90	9.5—10.2	8.50	8.4—8.6
Nagpur	7.37	7.1—7.7	6.93	6.8—7.1
Kanpur	12.33	11.9—12.5	8.47	8.4—8.5
Ferozepur	10.50	10.3—10.8	8.40	8.3—8.5
Poona	11.10	10.8—11.3	8.40	8.3—8.5
Pusa	9.70	9.2—10.0	8.10	7.9—8.3
Delhi	10.30	10.2—10.4	6.93	6.9—7.1

\* After deduction of blanks and that contained in the soil extracts.

TABLE IV.

*Effect of water extract of a Delhi soil on nitrogen fixation by Azotobacter sp.*

Ml. of soil extract/100 ml. of medium	Nitrogen fixed (unaltered extract (mg./g. of mannite)		Nitrogen fixed (organic matter free extract (mg./g. of mannite)	
	Average	Range	Average	Range
0	5.43	5.3—5.6	6.20	6.1—6.3
1	6.07	5.9—6.2	6.10	6.0—6.2
5	7.87	7.6—8.1	6.30	6.2—6.4
10	8.93	8.8—9.0	6.17	6.1—6.2
20	8.97	8.9—9.0	6.97	6.9—7.0
40	9.27	9.1—9.4	6.83	6.7—7.0
80	11.50	11.0—11.8	6.40	6.3—6.5
100	11.10	11.1—11.2	6.40	6.0—6.7

## SUMMARY

Water extracts of soils obtained by both hot and cold extraction, exerted a stimulating effect on fixation of nitrogen by *Azotobacter sp.* Extracts obtained by hot extraction caused higher fixation of nitrogen than extracts obtained by cold extraction. This is because of the presence of phosphorus in the former extract. Inorganic salts in soil extracts exerted a depressing effect while organic matter had a stimulating effect on nitrogen-fixation.

## ACKNOWLEDGMENT

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# INDIAN JOURNAL OF MICROBIOLOGY

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## EXCRETION OF CERTAIN AMINO-ACIDS DURING THE INHIBITION OF GROWTH OF *VIBRIO CHOLERA*E BY DIFFERENT ANTIVITAMINS

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A large number of bacteria have been found to accumulate amino acids in their medium (Gale, 1953; Britten *et al.*, 1955; Cohen, 1956), but the effect of different antivitamins on the accumulation has not been studied. Holden (1959) has observed that there is no difference in the glutamate accumulation in the B6 deficient and normal cells of *Lactobacillus arabinosus*. Friedman and Moat (1958) have found that in biotin-deficient yeast there is blocking in the synthesis of purines and this blocking can be reversed by the addition of aspartic acid to the medium. Webb (1958), studying on *Aerobacter aerogenes*, has shown that during the aminopterin inhibition of this organism, there is accumulation of alanine and valine. In this communication, the effects of oxythiamine,

pyrithiamine, desoxypyridoxine, pantooyltaurine and aminopterin on the growth of various strains of *Vibrio cholerae* and also on the accumulation of amino acids during inhibition have been presented.

## MATERIALS AND METHODS

### *Growth medium*

The action of different antivitamins on the growth of *V. cholerae* has been studied using peptone-water medium as well as glucose-ammonium phosphate media of Saxena *et al.* (1953). The excretion of amino acid has, however, been determined in the Saxena's medium using an *Ogawa* strain.

### *Measurement of growth*

The growth of eight different strains of *Ogawa*, one *Inaba* and one *El Tor vibrio* has been followed turbidimetrically using a 540 m $\mu$  filter in a Klett-Summerson photo-electric colorimeter. The results have been checked frequently by simultaneous viable count.

### *Antivitamins*

Pyrithiamine, oxythiamine, desoxypyridoxine and pantooyltaurine were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A. and aminopterin was obtained from the Lederle Laboratories, U.S.A. The antivitamins were used at a concentration of 100  $\mu$ g./ml. of the incubating medium.

### *Identification of amino acids in the culture medium*

Direct chromatography in solvent system, butanol, acetic acid and water (4:1:1), of culture supernatants from various strains of *V. cholerae* grown for 18 hr. in Saxena's medium containing different antivitamins, after desalting by methanol according to the method described by Webb (1958), has been made to determine the excretion of amino acids by the organism in the culture medium.

## RESULTS

It has been found that the inhibition is more consistent ranging from 30 to 75% with different antivitamins, using Saxena's medium (Table 1). The defect of this medium is that the growth rate of *V. cholerae* is very slow and in order to get an appreciable growth, incubation for about 18 hr. is necessary. The accumulation of amino acid during inhibition has also been studied using this medium. In peptone-water medium an appreciable growth of the control cultures has been found even after 3 hr. In order to determine the phase at which the growth inhibition is maximum, the growth in the presence of antivitamins has been measured after 3, 6 and 18 hr. incubation in peptone-water medium. The inhibition percentage has been found to decrease with increase in the period of incubation. It has also been observed (Table 1) that the addition of antivitamins to the culture media after 3 hr. of growth and subsequent incubation upto 6 hr. or even upto 18 hr. causes almost the same type of growth inhibition compared to the inhibition observed when antivitamins were added from the beginning.

TABLE I.

*Effect of certain antivitamins on the growth of Vibrio cholerae*

Additions to the medium	Growth (Klett reading at 540 m $\mu$ )							
	Saxena's medium, grown for 18 hr.*				Peptone-water medium, grown for 6 hr.**			
	Ogawa	El Tor	Ogawa I	Ogawa II	El Tor I	El Tor II	Inaba I	Inaba II
None (control)	40	37	31	37	57	61	32	23
Oxythiamine	19	20	24	26	30	38	16	13
Pyrithiamine	16	26	15	23	42	36	18	13
Desoxypyridoxine	18	20	13	18	31	39	14	12
Pantoyltaurine	20	12	13	18	36	36	12	14
Aminopterin	32	20	25	28	37	42	20	18

\* No growth was observed with Inaba in Saxena's medium after 18 hr. incubation.

\*\*In I, the antivitamins were added from the beginning and in II they were added after initial 3 hr. of growth.

Chromatography of culture supernatants from various strains of *V. cholerae* grown for 18 hr. in Saxena's medium containing different antivitamins, after desalting by methanol (Webb, 1958), has revealed that the accumulation of alanine and aspartic acid takes place with all the antivitamins except with oxythiamine or pyrithiamine, where besides alanine and aspartic acid, glutamic acid was found to accumulate to a large extent. Very faint spots for alanine and also for aspartic acid have been detected in the filtrates of control cultures incubated for the same period in the absence of the antivitamins.

## DISCUSSION

*Vibrio cholerae* has a very high transaminase activity (Saxena *et al.*, 1956). The accumulation of amino acids such as alanine, aspartic acid and also glutamic acid in the case of thiamine antagonists and only alanine and aspartic acid in the case of other vitamin analogues during the inhibition of growth in the presence of antivitamins might be due to the partial blocking of the amino acid metabolism. Since these amino acids are also involved in the synthesis of nucleotides, their accumulation might also be due to the partial blocking in the nucleic acid synthesis, thereby resulting in inhibition of growth.

## SUMMARY

The antivitamins such as oxythiamine, pyrithiamine, desoxypyridoxine, pantoyltaurine and aminopterin have been found to produce marked growth-inhibition of different strains of *Vibrio cholerae* when added to the medium during their growth. The addition of these antivitamins individually except oxythiamine and pyrithiamine, brings about an increased excretion of alanine and aspartic acid only, whereas in the case of oxythiamine and pyrithiamine, the excretion of glutamic acid besides alanine and aspartic acid has been observed.

## ACKNOWLEDGMENT

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# INDIAN JOURNAL OF MICROBIOLOGY

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## OCCURRENCE OF A STRAIN OF TURNIP CRINKLE VIRUS IN INDIA

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During 1958, turnip plants grown in two plots near Lucknow University were found affected by crinkle disease. The leaves presented rugged appearance, developed yellow patches and were brittle. The yellow patches coalesced and became necrotic with the age of the plants. In course of about a month, the affected leaves began to die and wither away. The growth of the diseased plants was much arrested and they were rosette in appearance.

### MATERIALS AND METHODS

Every g. of diseased leaves was crushed with 1 ml. of sterile distilled water and the extract was centrifuged for 15 min. at 3,000 r.p.m. Plants were inoculated with pale brown supernatant liquid and were kept in insect-proof conditions. Carborundum powder was used as an abrasive.

### RESULTS

The disease was successfully transmitted by means of mechanical inoculations but attempts to transmit it through aphids (*Myzus persicae* Sulz.) and cuscute (*Cuscuta reflexa* L.) were not successful.

### HOST RANGE OF THE VIRUS

#### Turnip (*Brassica rapa* L.)

Disease symptoms appeared with yellow spots which soon coalesced to give chlorotic appearance to the leaves with necrosis. Later, the leaves crinkled and withered away. Leaves emerging after inoculation were diseased and the plant became rosette in appearance with stunted growth (Plate I, Fig. 1.)

Cauliflower (*B. oleracea* var. *botrytis* L.)

The most pronounced symptoms of the disease were curling and vein banding, mostly in young leaves and growth of the plants were arrested.

Cabbage (*B. oleracea* var. *capitata* L.)

Mottling with the appearance of minute chlorotic or necrotic lesions was observed. The growth of the plant was retarded after infection (Plate I, Fig. 2A).

Kohlrabi (*B. oleracea* L. var. *caulorapa* Pasq.)

This plant was highly susceptible. The leaves puckered or curled with blistering and necrosis. The growth of the plant was also retarded after infection (Plate I, Fig. 2B.)

The symptoms developing on plants, belonging to the families other than cruciferae, are summarized in Table I.

TABLE I.  
Showing symptoms developing on plants

Family	Host Plant	Characteristic symptoms.
Solanaceae	<i>Nicotiana tabacum</i> L. var. Turkish	Inoculated leaves became slightly chlorotic with necrotic patches. Very young leaves emerging after infection remained thin and yellowish.
,,	<i>N. plumbaginifolia</i> L.	Slight chlorosis of leaves. New leaves emerging after infection remained narrow, fragile and yellowish.
,,	<i>N. glutinosa</i> L.	Leaves puckered and occasionally developed faint vein banding.
,,	<i>Lycopersicon esculentum</i> Mill.	Chlorosis immediately followed by necrosis; the whole compound leaf with rachis tended to curl downward. The disease was systemic.
,,	<i>Solanum tuberosum</i> L.	The symptoms resembled those on tomato with the difference that the whole leaf did not tend to curl downward. Necrosis was not severe.
,,	<i>S. nigrum</i> L.	Chlorosis followed by necrosis.
,,	<i>Datura stramonium</i> L.	Fairly visible light chlorotic lesions; the margins of leaves were slightly curled inward.
Leguminosae	<i>Cymopsis tetragonoloba</i> (L) Taub.	Minute necrotic lesions appeared on the leaflets after inoculation, non inoculated halves remained normal.
Chenopodiaceae	<i>Beta vulgaris</i> L.	Sometimes slight curling and mottling of leaves was observed.

Necrotic local lesions were not observed on the inoculated leaves of *Gomphrena globosa* L. and *Chenopodium amaranticolor* Costs and Reyn.

*Physical properties of virus**Dilution end point*

The standard extract was diluted with sterile distilled water and six young vigorously growing turnip, cauliflower, kohlrabi and cabbage plants were inoculated with each dilution. The number of plants infected with various dilutions is given in Table II. Positive infections were obtained regularly upto the dilution of  $10^{-5}$  but not at  $10^{-6}$ .

*Thermal inactivation point*

This was determined by heating 2 ml. samples of standard extract on a water bath for 10 min. at various temperatures. The virus becomes inactivated between  $85^{\circ}\text{C}$ — $90^{\circ}\text{C}$ .

TABLE II.  
*Dilution end point of the virus in the extract*

Dilution of the extract	Turnip	Cauli-flower	Kohlrabi	Cabbage
1/10	6	5	6	6
1/100	6	5	6	5
1/1,000	5	2	5	3
1/10,000	4	2	3	1
1/1,00,000	2	1	1	1
1/10,00,000	1	Nil	Nil	Nil

*Longevity of the virus in vitro*

The virus in the sap was infective for about 2 weeks at room temperature ( $25^{\circ}\text{C}$  —  $30^{\circ}\text{C}$ ).

*Serological reaction*

Antiserum of the virus reacted with antigen upto the dilution of 1/160 or more. 1/10 dilution of the antiserum gave visible coarse granular precipitate upto  $10^{-5}$  dilution of antigen and this was the end point.

## DISCUSSION

Since Gardner and Kendrik (1921) reported "turnip mosaic", the first cruciferous virus disease, Clayton (1930), Hoggan and Johnson (1935), Tompkins and Thomas (1938), Walker *et al.* (1945), Sylvester (1953) and several others have studied various virus diseases of cruciferous plants. All of them were found to be transmissible by aphids. Markham and Smith (1949) described turnip yellow mosaic virus (TYMV), the first virus attacking crucifers, which was not transmitted by aphids but was transmitted by flea-beetles. The virus disease investigated here, although not transmissible by aphids, is not related to turnip yellow mosaic virus because of marked differences in host reactions, host range and resistance to heat. Broadbent and Heathcote (1957) have reported turnip

crinkle virus, which was also not transmissible by aphids. The virus described by us resembles this virus in certain properties i.e. host reactions, host range and dilution end point but seems to be a new strain because of the differences in thermal death point, transmissibility to *Nicotiana* spp. and failure to produce necrotic local lesions on *G. globosa* L. and *C. amaranticolor* Costs and Reyn.

#### SUMMARY

A virus disease of turnip (*Brassica rapa* L.) has been described. It is characterised by crinkling, stunting and rosetting of plants and is readily transmissible by mechanical means but not by aphids. The virus has a dilution end point of  $10^{-5}$  and thermal inactivation point between  $85^{\circ}\text{C}$ — $90^{\circ}\text{C}$ . It can infect cauliflower (*B. oleracea* L. var. *botrytis* L.), kohlrabi (*B. oleracea* L. var. *caulorapa* Pasq.), Cabbage (*B. oleracea* L. var. *capitata* L.), tobacco (*Nicotiana* spp.), tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), nightshade (*S. nigrum* L.), datura (*Datura stramonium* L.), guar (*Cymopsis tetragonoloba* L. Taub.) and sugarbeet (*Beta vulgaris* L.). The virus appears to be a new strain of turnip crinkle virus.

#### ACKNOWLEDGMENT

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EXPLANATION OF PLATES  
Plate I

*Diseased and healthy turnip plants. Yellowing and crinkling of leaves and stunted growth of diseased plant are clearly visible.*



- A. Diseased cabbage plant with minute chlorotic lesions. Some of the lesions have coalesced along the veins.
- B. Diseased kohlrabi plant. Leaf lamina has curled and shrunk with blistering.



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## THE EFFECT OF POTASSIUM NUTRITION ON MULTIPLICATION OF TOBACCO MOSAIC VIRUS IN TURKISH TOBACCO (*NICOTIANA TABACUM* L.) PLANTS

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Various workers have studied the effect of nitrogen, phosphorus and potassium and such minor elements as zinc, manganese and iron on virus multiplication in plants. The effect of nitrogen has been studied more intensively than that of potassium (Cheo *et al.*, 1952; Pound and Weathers, 1953; Weathers and Pound, 1954; Bawden and Kassanis, 1950; Spencer, 1939; 1941) on virus activity. The present paper deals with the effect of different levels of potassium on height, fresh and dry weights and virus concentration of the Turkish tobacco (*Nicotiana tabacum* L.) plants systemically infected with tobacco mosaic virus.

### MATERIALS AND METHODS

Six-inch clay pots were painted inside with bitumen paint, drainage holes plugged with glass wool and covered with watch glasses. They were filled with purified sand (Hewitt, 1946) and housed in insect-proof glass house. Arnon and Hoagland's (1940) nutrient solution was added to the sand and seeds of Turkish tobacco (*N. tabacum* L.) were sown. After 20 days the seedlings grew to a 3-4 leaf stage. Seedlings of uniform size were selected and each was transplanted in a separate pot. The pots were grouped into 4 batches, 30 pots in a batch, and were irrigated twice a week with Arnon and Hoagland's solution containing 3,900, 390, 39 and 0 ppm of potassium ( $\text{KNO}_3$ ). Sufficient solution was added to allow free flow of drainage. The accumulation of salts was checked to a considerable extent by flushing the pots with distilled water.

Twenty days later, when potassium deficiency symptoms became clear in batch receiving 0 ppm of potassium, tobacco mosaic virus was inoculated into all the four batches of plants. Two or three lower leaves per plant were inoculated using carborundum powder as an abrasive. Control plants were not inoculated.

At 0, 15 and 30 days after inoculation, 4 plants from each batch of inoculated and 4 plants from the control were uprooted and their mean length in cm., fresh and dry weights in g. were determined. The dry weight, without the roots, was determined after heating the plants for 10 hr. at  $100^\circ\text{C}$  and then at  $80^\circ\text{C}$  till the weight was constant. Rest of the plants were homogenised with distilled water, in the proportion of 1 ml. of distilled water to one g. of wet plant tissue, in pestle and mortar. The juice was

expressed through muslin cloth and stored in a deep freeze. For assay of virus concentration in the juice, counts of local lesions produced on half leaves of *N. glutinosa* L. were taken. The juice was heated to 60°C for 10 min. and centrifuged at 3,000 r.p.m. for 15 min. before inoculation on the leaves. For optical density readings by Unicam spectrophotometer (Takahashi, 1951) a quantity of the expressed sap was alternately centrifuged at low (3,500 r.p.m.) and high (12,000 r.p.m.) speed for 15 min. in angle centrifuge and the supernatant was diluted 1/100 in distilled water.

Total nitrogen content of the heat clarified sap was determined by the micro-kjeldahl method.

The experiment was conducted during the months of December and February. The temperature inside the glasshouse was in the range of 20°C — 30°C.

## RESULTS

Height, fresh and dry weights of the diseased and healthy plants supplied with different concentrations of potassium are presented in Tables I — III. When the concentration of potassium was increased or decreased beyond 390 ppm, adverse effects on height, fresh and dry weights of Turkish tobacco plants, both infected and uninfected, were observed (Tables I — III; Plate I, Figs. 2 and 3). The fresh and dry weights of the infected plants fell considerably below normal on the 50th day. The height was not so much affected.

TABLE I.

*Effect of potassium on the height of healthy and diseased plants of Turkish tobacco (N. tabacum L.)*

Potassium	Interval (days)*					
	20		35		50	
	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
0 ppm	6.3	6.3	10.5	8.0	19.2	16.6
39 ppm	8.8	8.8	15.0	13.0	59.7	36.5
390 ppm	12.5	12.5	27.5	16.0	68.5	40.3
3900 ppm	10.5	10.5	23.5	14.0	49.3	36.4

\* Time between transplantation and harvest of plants. The figures represent average height of 4 plants expressed in cm.

The most pronounced symptoms of potassium deficiency were interveinal chlorosis and scorching of margins and tips of older leaves (Plate I, Fig. 1). Tobacco mosaic virus infection in those plants was quite indistinct (Plate I, Fig. 3), the leaf size was not drastically reduced.

Increase beyond 390 ppm of potassium resulted in the decrease in the amount of active virus produced by the plants. Plants grown under potassium deficient conditions (0 ppm) produced very little virus (Table IV) and the growth was also stunted,

TABLE II.

*Effect of potassium on fresh weight of healthy and diseased plants of Turkish tobacco (N. tabacum L.)*

Potassium	Interval (days)*					
	20		35		50	
	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
0 ppm	0.49	0.49	1.31	0.80	6.98	6.78
39 ppm	1.17	1.17	9.94	3.62	43.14	12.78
390 ppm	2.65	2.65	16.93	13.40	100.78	16.88
3900 ppm	2.45	2.45	17.47	6.69	44.38	15.35

\* Time between transplantation and harvest of plants. The figures represent average weight of 4 plants expressed in g.

TABLE III.

*Effect of potassium on dry weight of healthy and diseased plants of Turkish tobacco (N. tabacum L.)*

Potassium	Interval (days)*					
	20		35		50	
	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
0 ppm	0.04	0.04	0.16	0.10	0.82	0.77
39 ppm	0.11	0.11	0.91	0.41	4.62	1.42
390 ppm	0.19	0.19	1.28	1.29	10.22	1.92
3900 ppm	0.17	0.17	1.36	0.58	4.45	1.81

\* Time between transplantation and harvest of plants. The figures represent the average weight of 4 plants expressed in g.

TABLE IV.

*Effect of potassium on the active virus formation in diseased plants of Turkish tobacco (N. tabacum L.)*

Potassium	Interval (days)*						
	20		35			50	
	Dilution			Dilution			
	—	1/10	1/100	1/1000	1/10	1/100	1/1000
0 ppm	—	6	3	2	8	3	2
39 ppm	—	315	138	61	733	411	149
390 ppm	—	295	112	52	741	465	172
3900 ppm	—	267	110	56	550	397	138

\* Time between transplantation and harvest of plants. The figures represent local lesions produced by heat clarified sap inoculated on 30 half leaves.

Optical density and total kjeldahl-nitrogen of heat clarified sap of infected plants increased with the increasing concentration of potassium upto 390 ppm. Beyond this, there was a decrease both in optical density and total kjeldahl-nitrogen in diseased plants (Table V).

TABLE V.

*Effect of potassium and virus infection on optical density and kjeldahl-nitrogen (total) of sap of diseased plants of Turkish tobacco (N. tabacum L.)*

Potassium	Interval (days)*				
	20	35	50		
		Optical density	Kjeldahl- nitrogen mg./ml.	Optical density	Kjeldahl- nitrogen mg./ml.
0 ppm	—	0.272	0.476	0.328	0.476
39 ppm	—	0.350	1.40	0.555	1.19
390 ppm	—	0.390	1.681	0.530	1.09
3900 ppm	—	0.342	1.232	0.490	0.910

\* Time between transplantation and harvest of plants. Optical density readings were taken at 265 m $\mu$  wave length and 1 cm. light path using Unicam spectrophotometer.

## DISCUSSION

Bawden and Kassanis (1950) conducted experiments with *N. tabacum* L. var. white burley, raised in a mixture of infertile soil, sand and peat and supplied with K<sub>2</sub>SO<sub>4</sub>. They suggested that potassium slightly reduced the TMV concentration of plant sap though it usually increased plant size and total virus content per plant. Cheo *et al.* (1952), on the other hand, showed that the growth (fresh weight) of *Spinacia oleracea* L. (spinach) plants, grown in washed quartz sand, was the highest at 430 ppm of potassium and there was pronounced stunting above and below this level. Rate of formation of cucumber virus 1 in spinach plants coincided with its growth pattern. Pound and Weathers (1953) reported that growth (fresh weight) of *N. glutinosa* L. and *N. multivelvis* Lindl., grown in washed sand, was maximum at 78 and 704 ppm of potassium respectively. The concentration of turnip virus 1 in these plants was also highest under these conditions.

Our results show that the severity of the virus symptoms followed closely the growth of the plants. Concentration of TMV in plant sap was not proportional to the growth. Plants supplied with 39 and 390 ppm of potassium, although differed in growth, produced almost the same concentration of virus. Similar was the case with optical density and total kjeldahl-nitrogen. Our findings, within the limits, agree with those of Bawden and Kassanis (1950) and Cheo *et al.* (1952).

In our experiments very little virus was formed during the 30 days at 0 ppm of potassium and it was highest at 390 ppm. Beyond this the virus formation was inhibited. In this respect potassium does not behave like phosphorus and nitrogen which are known

to promote virus protein synthesis under the conditions of high nutrient supply, even though growth is adversely affected (Bawden and Kassanis, 1950; Selman and Grant, 1957; Spencer, 1939; 1941).

Effect of potassium nutrition on plant metabolism has been reviewed by Thomas *et al* (1955). Potassium is a mobile element which accumulates easily in leaves and the meristematic cells; vegetative developments are favoured by abundant supply. Further, potassium nutrition slightly enhances the water content per unit area of leaf. It is probable that virus activity is affected by differences in potassium supply, by upsetting one or all of these vital metabolic processes.

#### SUMMARY

The effect of different levels of potassium, on height, fresh and dry weights of healthy and tobacco mosaic virus infected Turkish tobacco (*Nicotiana tabacum* L.) plants was studied. When the concentration of potassium was increased or decreased beyond 390 ppm, adverse effect, both in infected and control plants, was observed. The fresh and dry weights of infected plants fell considerably below normal but the height was not much affected. Increase of potassium beyond 390 ppm resulted in slightly decreased virus production in plants. Optical density and total kjeldahl-nitrogen of heat-clarified sap of infected plants increased with the increasing concentration of potassium upto 390 ppm after which there was a decrease in both.

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## EXPLANATION OF PLATES

## Plate I -

## FIGURE 1.

*Potassium deficiency symptoms in leaves of Turkish tobacco plants. There is interveinal chlorosis and scorching of tips and margins.*

## FIGURE 2.

*Turkish tobacco plants, 50 days after transplantation, from left to right showing the effect of 3900, 390, 39 and 0 ppm of potassium.*

## FIGURE 3.

*Effect of potassium on TMV infected Turkish tobacco plants. Plants, 50 days after transplantation from left to right receiving 3900, 390, 39 and 0 ppm of potassium.*





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## STUDIES ON THE GERMINATION OF BACTERIAL SPORES

### I. HEAT RESISTANCE OF *BACILLUS SUBTILIS* SPORES DURING INCUBATION IN PEPTONE AND GLUCOSE SOLUTIONS

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Subba Rao (1959) found that about 33% of the spores of *Bacillus subtilis* undergo changes during their contact with peptone in the presence of formaldehyde and become more susceptible to the bactericidal activity of formaldehyde, even in lower concentrations. Since it has been reported that incubation of bacterial spores in nutritive media results in the loss of their heat resistance (Curran, 1931; Hills, 1949), a detailed study of the effect of incubation of the bacterial spores in solutions of different substances has been taken up. The results obtained with peptone and glucose solutions are presented in this paper.

#### MATERIALS AND METHODS

##### *Spore suspension*

*B. subtilis* was obtained from the National Collection of Industrial Micro-organisms, Poona. The surface growth of 10 days old culture on agar slope was washed off with 20 ml. of water. The spores and the vegetative cells were washed by centrifuging. They were suspended in 20 ml. of water and distributed into 1 ml. ampoules and sealed. The ampoules were heated in a water bath at 80°C for 5 min. to destroy any vegetative cells and stored in a refrigerator.

##### *Viable count of spore suspension*

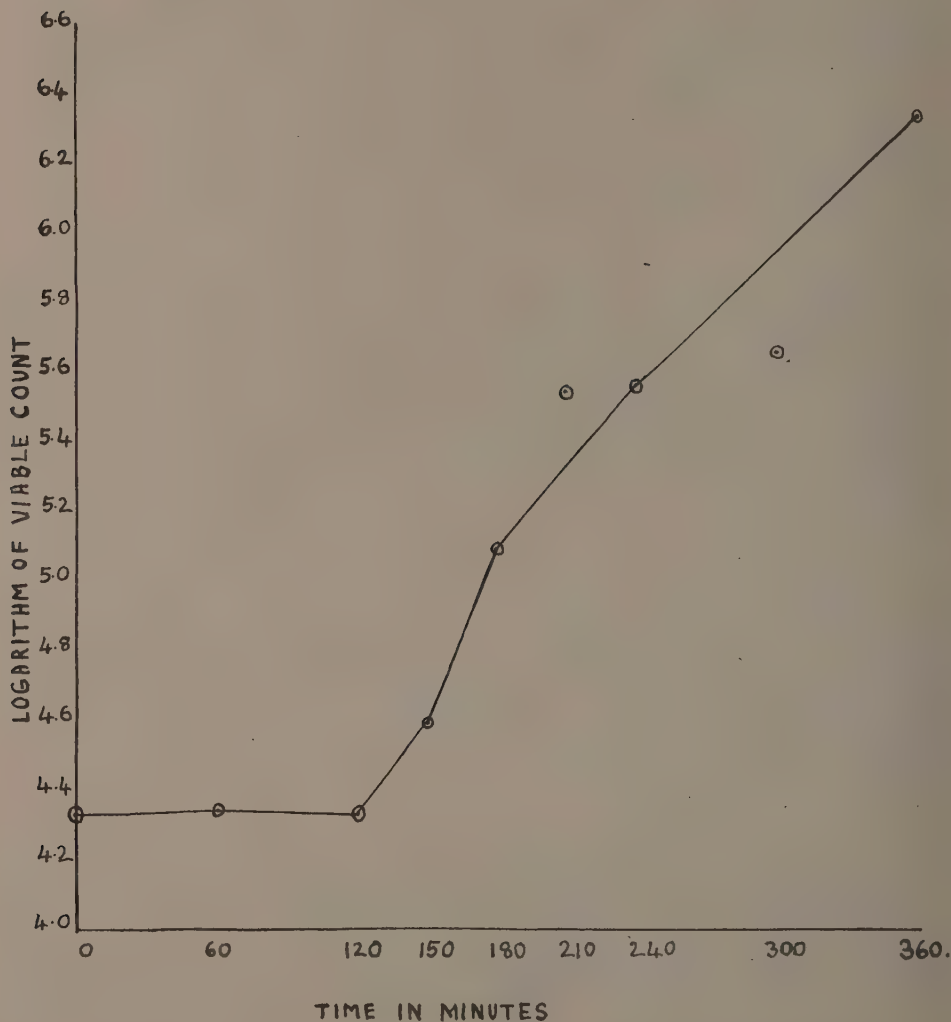
One ml. of the spore suspension containing 200,000 spores was added to 9 ml. of 1% peptone (oxoid) solution and the mixture was incubated at 37°C. 1 ml. samples were withdrawn at the end of 30 min. intervals and 1 ml. of 10<sup>-2</sup> dilutions was inoculated into each of 5 roll-tubes containing 5 ml. of molten agar maintained at 45°C. They were immediately rolled under a stream of cold water until the medium had set and incubated for 48 hr. at 37°C. The average count of five tubes was taken as the number of organisms surviving.

##### *Loss of heat resistance of spores*

Loss of heat resistance of spores was determined by heating 10<sup>-1</sup> dilution of the spores in a water bath at 80°C for 5 min. to destroy the organisms that had undergone

changes and lost their heat resistance during incubation. The number of spores surviving was counted as described above. The difference between the original count and count obtained after heat treatment gave the number of spores that lost their heat resistance.

FIGURE 1



LOGARITHM OF VIABLE COUNT VS. TIME IN MINUTES DURING THE  
INCUBATION OF *B. SUBTILIS* SPORES IN 1 PERCENT PEPTONE SOLUTION.

TABLE I

*Effect of incubation in 1% peptone, 1% glucose and a mixture containing 1% peptone and 1% glucose on the heat resistance of B. subtilis spores.*

Incuba- tion time min.	1 % peptone solution					1 % glucose solution					Mixture containing 1 % peptone and 1 % glucose				
	% survivors after heat resistance test					% survivors after heat resistance test					% survivors after heat resistance test				
	EXPERIMENT					EXPERIMENT					EXPERIMENT				
	1	2	3	Average	% of spores lost heat	1	2	3	Average	% of spores lost heat	1	2	3	Average	% of spores lost heat
0	—	—	—	—	0	—	—	—	—	0	—	—	—	—	0
30	26.0	31.0	21.2	26.1	73.9	93.05	94.1	103	96.72	3.28	16.0	27.6	21	21.5	78.5
60	9.2	7.1	11.5	9.3	90.7	93.05	87.8	102	94.28	5.72	2.9	3.7	9	5.2	94.8
90	5.3	5.0	4.8	5.0	95.0	84.9	93.2	97.1	91.7	8.3	2.2	2.4	4	2.9	97.1
120	3.0	3.0	3.0	3.0	97.0	88.4	96.2	102	95.5	4.5	0.7	0.6	2	1.1	98.9
150	3.0	0.8	2.4	2.1	97.9	82.6	83.6	86.2	84.1	15.9	1.4	0.6	1	1.0	99.0
180	1.5	2.0	2.0	1.8	98.2	88.4	87.8	99.1	91.8	9.2	1.4	0.6	1	1.0	99.0

## RESULTS

The mean viable counts increased from  $21.1 \times 10^3$  to  $22.15 \times 10^5$  during incubation in 1% peptone from 0 to 6 hr. and the logarithms of these counts are plotted against time in Fig. 1. The viable counts of the spores remained constant during 120 min. of incubation and then began to increase.

The effect of incubation in 1% peptone, 1% glucose (A.R.) and a mixture containing 1% peptone and 1% glucose on the heat resistance of *B. subtilis* spores is given in Table 1. It would be seen that nearly 90% of the spores lost their heat resistance within 60 min. of incubation with 1% peptone. During that period, only 5% of the spores lost their resistance when incubated in 1% glucose. In a mixture containing 1% peptone and 1% glucose, the results were similar to that obtained with 1% peptone.

## DISCUSSION

Curran (1931) and Hills (1949) reported that spores germinated in solutions containing small quantities of nutritive materials like broth, yeast extract and peptic or tryptic digests of casein. Loss of heat resistance was taken as a reliable test for spore germination by Wynne and Foster (1948) and this criterion was adopted by other workers in studies on viability of spores (Hills, 1949; Bullock and Tallentire, 1952).

The results reported here would show that *B. subtilis* spores lose their heat resistance within 60 min. of incubation in peptone and this finding is in conformity with the work of Curran (1931) and Keilin and Hartree (1947). The concentration of glucose employed in the present work did not hasten the germination and growth of *B. subtilis* spores as reported earlier by Powell (1951). Bullock *et al.* (1949) found difficulty in obtaining viable counts with vegetative cells of *B. subtilis*, since they have a tendency to form chains and matty growth. No such difficulty was experienced during the present work. By plating the suitable dilutions discrete colonies were obtained.

## SUMMARY

Ninety per cent spores of *Bacillus subtilis* lose their heat resistance within 60 min. of incubation in 1% peptone solution, while only 5% of them lose heat resistance in 1% glucose. The results obtained with a mixture of peptone and glucose were similar to that in peptone.

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# INDIAN JOURNAL OF MICROBIOLOGY

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## SOME OBSERVATIONS ON CONCENTRATION METHODS EMPLOYED FOR THE DEMONSTRATION OF TUBERCLE BACILLI MICROSCOPICALLY AND CULTURALLY

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Tubercle bacilli present in a specimen of sputum can be concentrated by a variety of methods—all designed to drive the tubercle bacilli present in a large volume of a given specimen into a much smaller volume and thereby to facilitate their easy demonstration by microscopic examination or their isolation by culture on a suitable medium. Sputum, normally, has a large number of micro-organisms which because they are fast-growing are capable of swamping out the slowly growing mycobacteria in artificial culture. Most of the concentration methods, therefore, are designed with the two-fold objective of (i) easy demonstration of acid-fast bacilli and (ii) their isolation by culture. For the latter, chemical reagents that have a lethal effect on the non-acid fast bacteria and a less harmful effect on the mycobacteria are employed. None the less most of the reagents used have got a deleterious, if sublethal, effect on the mycobacteria. It is necessary therefore that the time of exposure of the A.F.B. to that action should be as short as is necessary to ensure the suppression of organisms other than the tubercle bacilli. For the concentration of tubercle bacilli gravitational force is employed and a good high-speed centrifuge is essential. It is obvious that no amount of centrifuging will concentrate the micro-organisms if the sample is thick, ropy, viscous sputum. The thinner or more liquid the maestrum, the easier it will be to force the organisms to the bottom of the centrifuge tube. Concentration methods are valuable for the demonstration of tubercle bacilli when they are present in small numbers sparsely distributed.

When we first commenced work requiring the routine demonstration of tubercle bacilli by microscopic, cultural and/or animal inoculation methods, Nassu's modification of Jungmann's method for the concentration of sputum was adopted. The results obtained by us were considered unsatisfactory from the culture point of view because the number of positives obtained by microscopic examination of the stained deposit of the concentrates was greater than the number from which tubercle bacilli were isolated. The possibility that this was due to the action of the antibiotics with which the patients were being treated was considered and not wholly accepted. Demonstration of tubercle bacilli by animal inoculation could not be freely adopted. Animals were

expensive and not always readily available and were therefore reserved for special occasions, as for example, the examination of pus from a cold abscess, or for pleural effusions, or for ascitis fluid, etc. Different chemical reagents and different concentration methods were tried out. The observations presented in this article are part of the work carried out to evolve a concentration method which in our hands produce better results.

(1) *Concentration of tubercle bacilli using 1% sodium carbonate*

During the course of these trials, it was observed when equal quantities of 1% aqueous solution of sodium carbonate and sputum are mixed together and kept at 37°C for 18-24 hr., the sputum is homogenised and completely liquefied. We therefore decided to try it out. The following procedure was adopted:

Five ml. of the sputum to be examined were mixed with an equal amount of 1% aqueous solution of sodium carbonate in a small flask and kept in the 37°C incubator till the following day (18-24 hr.). The sample was then observed to be thoroughly liquefied. This was then centrifuged at 3,000 r.p.m for 10 min. The supernatant fluid was discarded and more sterile distilled water was added and the deposit resuspended in it by thorough mixing. It was then centrifuged again at 3,000 r.p.m for another 10 min. This procedure was repeated once again and an alkali free deposit was so obtained. This deposit was then suspended in 100 units of penicillin solution and seeded on to Löwenstein Jensen egg medium. Smear preparations were also made and stained by the Ziehl Nelsen method for A.F.B. It was noted that the deposit obtained was very little, due probably to the thorough liquefaction of the sputum so that only debris and organisms in enormous numbers went to make up the deposit. The inoculated L. J. medium bottles were incubated at 37°C. Most of them showed a profuse growth of non-acid fast organisms within 2-3 days of incubation, but a few that were not contaminated gave a very good growth of tubercle bacilli covering the whole surface of the medium. It was therefore concluded that the lethal effect of 1% sodium carbonate in water was poor and the method unsuited for use when isolation of T.B. by culture was intended. But the microscopic examination of the stained smears of deposit obtained by its use was so impressive that it was decided to compare it with the following methods:

Antiformin (Uhlenhuth and Xylander, 1909) sodium hyperchlorite (Jones, 1945), 6% sulphuric acid (Corper and Uyei, 1927), 4% sodium hydroxide (Petroff, 1915), 5% oxalic acid (Corper and Uyei, 1930), trisodium phosphate (Corper and Stoner, 1946), and Jungmann's reagents in accordance with that workers original method (Jungmann and Guschgat, 1938).

## METHOD

Sputum from tuberculous patients was thoroughly shaken with glass beads to obtain as uniform a distribution of tubercle bacilli as possible. Five ml. of the sample were then transferred into each of 8 small flasks, each to be treated according to one of the above methods. One ml. of distilled water was added to the final deposit obtained after centrifuging and washing as previously described and smears prepared with an equal quantity of each of them. These were examined after staining by the Ziehl Nelsen method and the number of A.F.B. present in 10 fields were counted. The procedure

was repeated in its entirety 10 times. It was found that the sodium carbonate method consistently gave the highest counts in that there were from  $1\frac{1}{2}$ -5 times as many bacteria as did any of the other smears prepared after treatment according to the other 7 methods. To simplify description, the count obtained by the sodium carbonate method was taken to represent 100% and the results of the others are shown as percentages of it. The figures are given in the following Table.

TABLE I

*Percentages of A.F.B. seen microscopically in smears stained by the Zeihl Nelsen method after concentration by one of the 8 methods mentioned in the text taking 100% for the sodium carbonate method*

$\text{Na}_2\text{CO}_3$	Anti-formin	Sod. hypochl	Petroff's	$\text{H}_2\text{SO}_4$	Oxalic acid	Trisodium $\text{PO}_4$	Jungman's reagents
100	20	65	60	25	30	40	25

A second comparative study was next undertaken. Sputum from cases of pulmonary tuberculosis was obtained. Each sample was distributed into two small flasks in 5 ml. amounts. One was treated with 1% sodium carbonate aqueous solution and the other with a digester containing NaOH 4%, pot. alum 0.2% and 0.02% brom thymol blue used for the concentration of sputum by the Hank's flocculation method (Hanks *et al.*, 1938). The flask containing the sputum and sodium carbonate was kept in the incubator 18-24 hr. the other was treated with an equal volume of digester and placed in a water bath at 37°C and occasionally shaken. When the digestion was completed it was neutralised with 25% by vol. concentrated hydrochloric acid. It was then shaken for 30 min. and stood for 5 min. on the work bench. Flocculation usually occurred but if it failed to do so 0.2 ml. of ferric chloride in water was added and the flask with its contents again shaken for about 5 min. It was then centrifuged for 5 min.; the supernatant was discarded, films were prepared by spreading 0.1 ml. of the deposit after it had been mixed with 0.5 ml. sterile distilled water. The flask containing the sodium carbonate solution was centrifuged the following day at the same speed as the other, its supernatant discarded, and films were prepared by spreading 0.1 ml. of the deposit after it had been mixed with 0.5 ml. sterile distilled water. All smear preparations were then stained with Ziehl Nelson stain, and examined microscopically by counting the number of A.F.B. present in 10 fields. The results obtained are given in Table II.

It is obvious from Tables I and II that the 1% sodium carbonate gives better results than any of the other 8 concentration methods with which it was compared. It is, therefore, felt that the sodium carbonate method is best for the examination of sputum that requires concentration for the demonstration of A.F.B. by microscopic examination. It should be the method of choice for small clinics and dispensaries where adequate facilities for the culture of tubercle bacilli are not available.

(2) *Observations on the gravitational force employed*

Once the sputum sample has been sufficiently liquefied the gravitational force applied by centrifuging, must be sufficient to force the A.F.B. present to the bottom of

TABLE II

*Showing the result of the experiment comparing the 1% sodium carbonate method with Hanks' flocculation method, for the demonstration of tubercle bacilli microscopically*

	F	C
Total number of specimens examined . . . . .	115	115
Number positive . . . . .	50	86
Number negative . . . . .	65	29
Number positive both methods . . . . .		50
Number negative both methods . . . . .		29
Number positive by C, negative by F . . . . .		36
Number negative by C, positive by F . . . . .		Nil

Hank's flocculation method indicated by F.  
1% sodium carbonate method indicated by C.

the centrifuge tube, within a specified maximum period of time depending upon the concentration method employed or else the deposit obtained may not be suitable for the cultivation of *Mycobacterium tuberculosis*.

The usual practice in this laboratory is that the homogenised specimen is centrifuged for 10 min. at 3,000 r.p.m. the supernatant is discarded and the deposit washed by adding sterile distilled water, resuspending the deposit by thorough mixing and centrifuging again at the same speed and for the same duration. This washing is repeated once again to ensure that the deposit to be seeded on to the medium is reasonably free from excess of the reagent used for homogenisation. The whole process from the addition of the homogenising reagent to the end of the first washing is to be completed within 30 min.

During the course of our investigation smears from the supernatant fluid to be discarded after the first centrifuging were prepared and examined microscopically after staining. Many A.F.B. were seen to be present lying separately or in twos. There were no clumps of mycobacteria of course. The following experiment was conducted, therefore, to determine the extent of the loss of viable mycobacteria through discarding.

Tubercle bacilli positive sputum was obtained and homogenised by Petroff's method (4% NaOH) and centrifuged at 3,000 r.p.m. for 10 min. The supernatant fluid was then transferred to a second centrifuge tube. The deposit was mixed with sterile distilled water and centrifuged again for 10 min at 3,000 r.p.m. The supernatant was again transferred to the tube containing the first lot. This was repeated once again except that the third centrifuging was done for 30 min. The supernatant was similarly collected. The fluid collected after the three centrifugations was centrifuged in an International refrigerated centrifuge at 18,000 r.p.m. at 4°C for 15 min. Smears from both the deposits (that obtained after 3,000 r.p.m. and the other at 18,000 r.p.m.) were prepared,

stained and examined for A.F.B. Also both the deposits were each seeded on to a set of 16 screw-capped bottles of Loewenstein-Jensen medium. The colonies in each set were counted after 8 weeks of incubation at 37°C. The result of this experiment, which was repeated 10 times is shown in Table III below:

TABLE III

*Number of colonies of tubercle bacilli in each set of 16 bottles of LJ medium seeded with deposit obtained (1) after centrifuging homogenised sputum at 3,000 r.p.m. and (2) the deposit obtained by centrifuging the supernatants at 18,000 r.p.m.*

		Deposit obtained after centrifugation at 3000 for 10 min.		Deposit obtained after centrifuging the supernatants at 18000 r.p.m. for 10 min.	
		820	Colonies	265	Colonies
1.	..	550	"	125	"
2.	..	800	"	180	"
3.	..	600	"	135	"
4.	..	810	"	190	"
5.	..	550	"	120	"
6.	..	770	"	105	"
7.	..	680	"	170	"
8.	..	740	"	210	"
9.	..	900	"	220	"
10.	..	Total:	7220	1720	"

TABLE IV

*Showing the results of the microscopic examination of stained smears prepared after concentration by Hank's flocculation method and the 1% NaCO<sub>3</sub> method employing varying centrifugal speeds*

	Number examined	Results expressed as percentages		
		Positive A.F.B.		Positive by C Negative by F
		F.	C.	
Hand Centrifuging ..	25	12	64	52
Centrifuging 3000 ..	65	38.5	74	35.4
Centrifuging 18000 ..	25	88	88	Nil.
Total:	115	43.5	74.7	31.3

Hank's flocculation method indicated by F  
1% sodium carbonate method indicated by C

It is seen from Table III that following the usual practice of centrifuging at 3,000 r.p.m. and discarding the supernatant fluid, viable tubercle bacilli are discarded which can produce approximately 24% more colonies of tubercle bacilli. This is a considerable loss and we therefore subjected sputum, which previous examination had shown to have only very few or no demonstrable A.F.B., to homogenisation with 4% NaOH for 10 min. and centrifugation at 18,000 r.p.m. and the usual washing with sterile distilled water. As most of the samples received in this laboratory for culture of T.B., have been first screened by repeated microscopic examination by clinics and found to be negative, centrifugation at 18,000 r.p.m. is now a routine procedure.

The effect of good liquefaction and centrifugation is also illustrated in the following table which gives results obtained by centrifuging for 5 min. homogenised sputum at varying centrifugal speeds—namely (1) in a hand centrifuge, (2) in an electrical centrifuge at 3,000 r.p.m. and (3) in a refrigerated centrifuge at 18,000 r.p.m. It was considered important that trials should be conducted after spinning in a hand centrifuge because the method of concentration with 1% sodium carbonate is recommended for small clinics where a hand centrifuge is often the only centrifuge available. Centrifuging for 5 min. was selected because it was difficult to manipulate a hand centrifuge at a reasonably good and uniform speed for more than 5 min.

It will be observed from Table IV.

- (1) that the carbonate method gives better results than Hank's flocculation method after centrifuging by all the three methods adopted and that the difference in positive findings is most marked in the results obtained by spinning in a hand centrifuge. This was considered to be due to the better liquefaction of the sputum sample by 1% NaCO<sub>3</sub> than that obtained by Hank's method.
- (2) that the percentage of positive findings increased with increased centrifugal speeds in that it rose from 12% obtained by hand centrifuging to 88% with very high-speed centrifuging.
- (3) that the difference of positive findings between the two methods of concentration disappear when adequate centrifuging is possible.

#### SUMMARY

Concentration of sputum using 1% sodium carbonate is recommended as the method of choice for those laboratories depending upon microscopic examination for the bacteriological diagnosis of tuberculosis.

When culture of tubercle bacilli is intended, especially for specimens containing few or no demonstrable tubercle bacilli, centrifugation in a refrigerated centrifuge at 18,000 r.p.m. after homogenising with 4% sodium hydroxide is recommended.

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# INDIAN JOURNAL OF MICROBIOLOGY

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## SURVEY OF *CANDIDA SPP.* IN EIGHT HUNDRED CASES OF LEUCORRHOEA

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Recently, mycotic infections have been found to be increasing at an alarming rate (Lee and Keifer, 1954). This is thought to be due to the use of broad-spectrum antibiotic-therapy (Bratland and Hotten, 1954; Brown *et al.*, 1953; Brown, 1954; Chaplan, 1955; Huppert and Cazin, 1955; Marconi, 1956; Sharp, 1954; Woods *et al.*, 1951). The present communication deals with the survey of *Candida spp.* in 800 cases of leucorrhoea.

### MATERIALS AND METHODS

Vaginal discharge of patients with leucorrhoea attending the outpatients-departments of the Hospitals of Chittaranjan Seva Sadan College of Obstetrics, Gynaecology and Child Health, were examined after smear-preparation and staining by Gram's method. Those cases which showed the presence of yeast-like fungi in the smear-examination and the development of colonies in Nickerson's medium were selected for further mycological studies. The morphological characteristics were also studied after staining by McGuire's technique as given by Dey (1958). For the study of cultural characteristics, the yeast-like fungi were grown in Nickerson's and Sabouraud's dextrose-agar media. Fermentation reactions were studied in glucose, maltose, sucrose and lactose sugar media. Chlamydospore formations were observed in human serum as a culture medium. The full description of this technique will be published elsewhere.

Albino mice (18-20 g.) were injected subcutaneously in the dorsum of the neck with saline suspension (0.25 ml./animal) of *Candida spp.* from Sabouraud's dextrose-agar media. There were 1-2 million organisms/ml. Smears from the site of injection were taken daily after making a nick with scalpel and were studied after staining. In all, 60 albino mice were used, 15 for each of the species, *C. albicans*, *C. tropicalis*, *C. krusei* and *C. stellatoidea*.

In rabbits, the injections were given per vein. The dose was 0.5 ml./animal. Four rabbits were used for *C. albicans*, 2 for *C. tropicalis*, 2 for *C. krusei* and 3 for *C. stellatoidea*.

In order to study the intercommunicability of the yeast-like fungi, the mouth of babies from mothers with vaginal candidiasis and the external genitalia of husbands were examined.

## RESULTS

Eight hundred cases of leucorrhoea were screened for infection by yeast-like fungi. Twenty-five per cent of these cases showed *Candida* spp. infection. *C. albicans* was found in 51%, *C. krusei* in 5%, *C. tropicalis* in 29%, *C. stellatoidea* in 13.5%, *C. pseudo-tropicalis* in 1% and *C. quilliermondi* in 0.5% of the cases. Gram-positive yeast-like fungi were detected in the vaginal smears of 178 patients. They existed in the form of spores, or well-developed mycelial filaments with typical septa and blastopores characteristic of *Candida* spp. In Nickerson's medium, from 200 cases, characteristic black or deep-brown colonies developed, proving the importance of taking cultures even during the preliminary investigation.

*C. albicans* in Nickerson's medium produced a peculiar type of colonies. They were black or deep brown in colour, size resembling a pin-head with spider-like process. These processes produced branching and sub-branching resembling roots of plants (Fig. 1). *C. tropicalis*, *C. krusei* and *C. stellatoidea* produced black or deep brown colonies, size varying from pin-head to pin-point but without radiating processes. The growth usually appeared within 72-96 hr. (Figs. 2, 3, 4).

All species of *Candida* produced creamy-white to pale-white colonies on Sabouraud's dextrose-agar medium.

In experiments with mice, *C. albicans* produced mycelia with spores and budding spores in the subcutaneous tissue (Fig. 5). Local abscess formation was also observed at the site of injection after 48 hr. On the 6th day, the skin over the site of injection sloughed out spontaneously, leaving an excavating ulcer (Fig. 6). On the 7th day, smears showed pus cells with spores and mycelia. The spores and mycelia diminished in size and number after the 7th day and completely disappeared by the 15th day, when only a few pus cells were found. Cultures were positive up to the 10th day. The ulcers were found to heal by the end of four weeks.

In *C. tropicalis*, *C. krusei* and *C. stellatoidea*, there were gradual development of spores and mycelia up to the 4th day after injection, but there was no ulcer-formation.

Rabbits were found to die within 4th to 6th day after injection with *C. albicans* and after 2 to 3 days with *C. stellatoidea*. In cases of *C. tropicalis* and *C. krusei*, none of the animals died.

Intercommunicability seems to be the characteristic feature of *C. albicans* and of no other species of *Candida*. The husbands (800) of the patients were screened; 15 had balanoposthitis due to *C. albicans*. Babies of patients showed mouth-infection of *C. albicans*. Out of 55 obstetric patients screened, 5 showed the development of thrush due to *C. albicans*.

## SUMMARY

Out of eight hundred cases of leucorrhoea screened for *Candida* spp. infection 25% were found positive. Culture of vaginal swab was observed to be the best method

for diagnosing *Candida* infection. *C. albicans* and *C. stellatoidea* were found pathogenic to mice and rabbits. *C. albicans* seems to be intercommunicable in human beings.

#### ACKNOWLEDGMENT

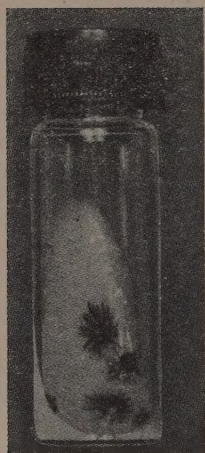
Our thanks are due to Dr. Subodh Mitra, Director and Principal of Chittaranjan Seva Sadan College of Obstetrics, Gynaecology and Child Health and Chittaranjan Cancer Hospital for his keen interest in this work. Our thanks are also due to Messrs. Sarabhai Chemicals, under whose grant-in-aid this work was carried out.

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#### EXPLANATION OF PLATES

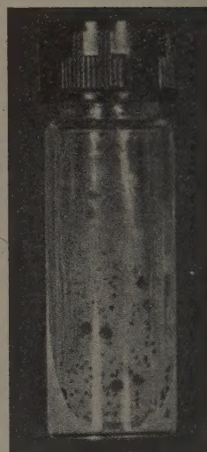
- FIG. 1. Growth characteristics of *Candida albicans*: 192 hr. growth in Nickerson's medium.  
FIG. 2. Growth characteristics of *C. tropicalis*: 192 hr. growth in Nickerson's medium.  
FIG. 3. Growth characteristics of *C. krusei*: 192 hr. growth in Nickerson's medium.  
FIG. 4. Growth characteristics of *C. Stellatoidea*: 192 hr. growth in Nickerson's medium.  
FIG. 5. Growth characteristics of *C. albicans*, *C. tropicalis* and *C. krusei*: 24 hr. growth in Sabouraud's dextrose-agar medium.  
FIG. 6. Development of spores and mycelia in the subcutaneous tissue of mice.



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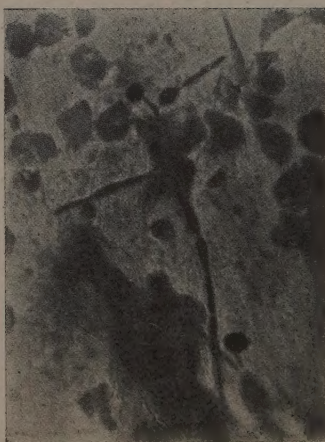
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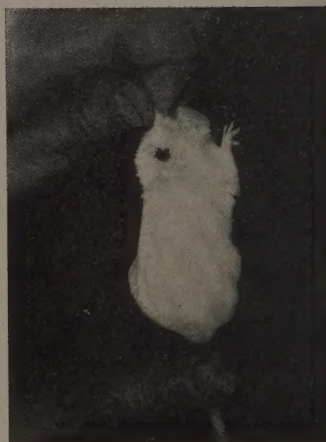
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